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Effect of Trypsin Treatment on the Heparin- and Receptor-Binding Properties of Human Plasma Low-Density Lipoproteins†

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ABSTRACT: The effect of trypsin treatment on the heparin- and receptor-binding properties of human plasma low-density lipoproteins (LDL) was examined. LDL were treated with trypsin (2% by weight) for 16 h at 37 °C, and the trypsinized core particles (T-LDL) were isolated by gel permeation chromatography on Sepharose CL-4B. Trypsin degraded the apolipoprotein B moiety (M_r = 550 000) of LDL into numerous peptides of M_r <110 000, resulting in the release of 25% ± 5% (n = 6) of its surface-associated protein. Relative to LDL, T-LDL had an increased phospholipid/protein ratio, decreased flotation density and α-helical structure, and increased fluidity of the surface and core constituents. Compared to LDL, T-LDL showed a 60% decreased capacity to suppress [1-^{14}C]acetate incorporation into cellular sterols consistent with decreased binding to the LDL receptor. In contrast, T-LDL showed an enhanced capacity to form soluble complexes with heparin in the absence and presence of 2 mM Ca^{2+}. Between 5 and 25 mM Ca^{2+}, both LDL and T-LDL were maximally precipitated by heparin; the stoichiometry of the insoluble complexes (uronic acid/phospholipid, w/w) was 0.054 ± 0.004 and 0.055 ± 0.005 (n = 18) for LDL and T-LDL, respectively. Thus, trypsin treatment significantly diminished the lipoprotein's interaction with cells but not with heparin. This finding suggests that proteolysis may decrease receptor-mediated uptake of LDL without diminishing the lipoprotein's reactivity with acellular components of the arterial wall.

Human plasma low-density lipoproteins (LDL)[1] are spherical micellar structures containing an outer monolayer of phospholipid and protein and an inner core of neutral lipids, primarily cholesteryl esters [see Morrisett et al. (1977) for a review]. There is great interest in understanding the structure and metabolism of LDL since their plasma levels are positively correlated with risk of coronary artery disease (The Lipid Research Clinics Coronary Primary Prevention Trial Results, 1984). The cellular catabolism of LDL occurs by their binding to specific high-affinity membrane receptors followed by the internalization and degradation of both protein and lipid (Brown & Goldstein, 1986); LDL–cholesterol is a major source of sterol for cell growth, maintenance, and steroid hormone production. LDL also bind glycosaminoglycans (GAG) of the extracellular matrix (Camejo, 1982). The interaction of LDL and GAG may play an important role in cholesterol deposition in the arterial wall and, thus, in the development of atherosclerosis (Hollander, 1976; Camejo, 1982).

It is generally accepted that apolipoprotein B (apoB), the major polypeptide of LDL, mediates the binding of the lipoprotein to membrane receptors and GAG; however, limited information is available on the details of these interactions. Although certain GAG, like heparin, release LDL from their cellular receptors (Goldstein et al., 1976), the structural relationship between the regions in apoB which mediate heparin binding and receptor binding is unknown.

The purpose of the present study was to compare the heparin- and receptor-binding properties of LDL and trypsin-treated LDL in order to define a possible relationship between

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[1] Abbreviations: LDL, low-density lipoprotein(s); GAG, glycosaminoglycan(s); apoB, apolipoprotein B; PMSF, phenylmethanesulfonyl fluoride; HRH, high reactive heparin; PBS, phosphate-buffered saline; DPH, 1,6-diphenylhexa-1,3,5-triene; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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these domains. The results show that trypsin treatment significantly reduces the binding of LDL to the high-affinity receptors on fibroblasts without diminishing their interaction with heparin, suggesting that different domains of apoB mediate receptor and heparin binding.

Materials and Methods

Isolation of LDL. Human plasma was obtained by plasmapheresis of normal fasting donors or subjects with familial type II hyperlipoproteinemia. Aprotinin (Sigma), phenylmethanesulfonyl fluoride (PMSF), sodium azide, and EDTA were added to final concentrations of 50 kallikrein inhibitory units/mL, 0.5 mM, 0.01%, and 1 mM, respectively. LDL were isolated between $d = 1.02$ and 1.05 g/mL by ultracentrifugal flotation in salt solutions of KBr as described previously (Cardin et al., 1982a). LDL were dialyzed against standard buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.01% NaN_3), stored at 4 °C under nitrogen, and used within 1 week. In some experiments, LDL were subjected to density-gradient ultracentrifugation between d = 1.006 and 1.37 g/mL. The gradient was formed with 6 mL of lipoprotein in standard buffer and 6 mL of standard buffer adjusted to $d = 1.37$ g/mL with KBr. After centrifugation for 20 h at 38 000 rpm and 15 °C in a Beckman SW-41 rotor, the samples were fractionated, and the refractive indexes were determined with a Bausch & Lomb ABBE-3L refractometer.

Tryptic Digestion of LDL. LDL (40 mg of protein in 10 mL of standard buffer) were incubated with 2% (w/w) bovine pancreatic trypsin (tosylphenyl chloromethyl ketone treated, Millipore) for 16 h at 37 °C; an identical sample without trypsin served as control. Trypsin-treated LDL and control LDL were adjusted to 1 mM PMSF and were then fractionated on a column (1.5 × 22 cm) of Sepharose CL-4B (Pharmacia). The column was equilibrated and eluted with standard buffer containing 1 mM PMSF. Effluent fractions were analyzed for protein and heparin binding (described below). The fractions corresponding to LDL and to the trypsinized (T-LDL) particles were pooled and concentrated in an Amicon ultrafiltration cell equipped with a PM-10 membrane. No residual trypsin activity was present in T-LDL as determined with Azocoll (Calbiochem) as substrate.

Heparin-Binding Assays. Insoluble complexes of heparin, LDL, and T-LDL were formed in the presence of 5–25 mM Ca^{2+}. For these experiments, total crude heparin from porcine intestinal mucosa (Hepar Industries, Franklin, OH) was fractionated on a column of LDL–AffiGel-10 as previously described (Cardin et al., 1984a). The fraction eluting with 0.5 M NaCl, designated high reactive heparin (HRH), was subsequently used. Increasing concentrations of LDL or T-LDL (0–150 μg of phospholipid/mL) were incubated with 30 μg of HRH (as uronic acid) in a total volume of 1.0 mL of standard buffer containing various amounts of $CaCl_2$. After a 30-min incubation at 25 °C, insoluble complexes were pelleted by low-speed centrifugation, and the supernatants were removed. Phospholipid in the pellets was determined by the method of Bartlett (1959), and uronic acid was determined by the carbazole reaction (Bitter & Muir, 1962). The amount of HRH in the insoluble complex was also determined by a filtration assay with ^{125}I-HRH; radioiodination of HRH was performed as described previously (Cardin et al., 1984d). LDL and T-LDL (0–150 μg of phospholipid/mL) were incubated with 30 μg of ^{125}I-HRH (as uronic acid) as described above. Insoluble complexes were collected by vacuum filtration on 0.45-μm Metricel filters (Gelman Filtration Products) and washed with 1.0 mL of standard buffer containing 5–25 mM $CaCl_2$ as appropriate. Filters were air-dried and quantitated for filter-retained ^{125}I by γ counting. Results were corrected for nonspecific binding of radioiodinated heparin.

Soluble complexes of HRH, LDL, and T-LDL were formed in the presence of 2 mM Ca^{2+}. Increasing concentrations of LDL or T-LDL (0–300 μg of phospholipid/mL) were incubated with ^{125}I-HRH in a total volume of 1.0 mL of standard buffer containing 2 mM Ca^{2+}. After incubation for 30 min at 25 °C, the samples were vacuum filtered on 0.025-μm Millipore filters, and the amount of filter-retained ^{125}I-HRH in the complex was quantitated by γ counting. In other experiments, soluble complexes of [^{3}H]heparin, LDL, and T-LDL were formed in the absence of Ca^{2+} and collected by vacuum filtration on 0.025-μm Millipore filters. LDL and T-LDL (100 μg of phospholipid/mL) were incubated with unfractionated [^{3}H]heparin (200 μCi/mg, New England Nuclear) in a total volume of 0.6 mL of standard buffer. The samples were incubated for 1 h at 25 °C and filtered, and the amount of filter-retained [^{3}H]heparin was determined by scintillation counting. Control experiments in the absence of lipoprotein and in the presence of cyclohexanedione-modified LDL (Noel et al., 1981) were performed to correct for the nonspecific binding of heparin.

LDL Receptor Assay. Human skin fibroblasts were grown, as described previously (Ranganathan et al., 1982a,b), in 35-mm culture dishes for 5 days. The cells were then incubated in Eagle's essential medium containing 10% lipoprotein-deficient serum for 24 h. The effect of LDL and T-LDL on the biosynthesis of cellular sterols was determined by the incorporation of [1-^{14}C]acetate into nonsaponifiable lipids (Ranganathan et al., 1982a). Each experiment was performed in triplicate, and the values are expressed as the mean ± SEM.

Other Methods. Molecular weights of apoB peptides were assessed by electrophoresis on 6–20% polyacrylamide gradient gels containing 1% sodium dodecyl sulfate and 6 M urea (Cardin et al., 1982a). Protein concentrations were determined by the method of Lowry et al. (1951) with BSA as the standard. Fluorescence polarization (p) measurements were conducted on a Perkin-Elmer MPF 44-A spectrofluorometer equipped with polarization accessory 063-0468 as described previously (Cardin et al., 1982b) and were calculated as $p = (V_v - L_v)/(V_v + L_v)$, where V_v and L_v are the fluorescence intensities measured with polarizers parallel and perpendicular to the vertically polarized exciting beam, respectively. Polarizations were determined at 2.0 °C intervals with the temperature maintained at ±0.1 °C by a Lauda-regualted water bath. Circular dichroic spectra were obtained with a Cary 61 spectropolarimeter operating at 25 ± 0.1 °C. LDL and T-LDL were adjusted to equal lipid concentrations; a value of 112 was used as the mean residue weight. Secondary structure was determined with a HELIX computer program (Thompson et al., 1976).

Results

Consistent with previous reports (Chapman et al., 1978; Triplett & Fisher, 1978), incubation of LDL with 2% (w/w) trypsin for 16 h at 37 °C resulted in the complete degradation of apoB (M_r 550 000) to yield peptides of <110 000 daltons. Trypsin-treated LDL and LDL (control) were chromatographed on Sepharose CL-4B, and the amount of lipid-free peptides was determined. As is shown in Figure 1A, LDL eluted from Sepharose CL-4B at fractions 17–32 (peak I), whereas the trypsin-treated sample (Figure 1B) contained additional material eluting in fractions 33–43 (peak II). Peak II contained no measurable lipid and accounted for 25% ± 5% ($n = 6$) of the total protein as small, water-soluble peptides.

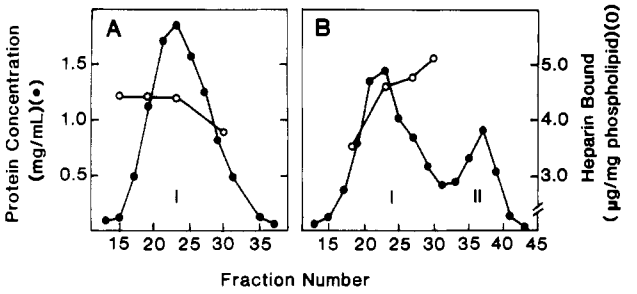

FIGURE 1: Chromatography of LDL (A) and trypsin-treated LDL (B) on Sepharose CL-4B. LDL (no enzyme control) and trypsin-treated LDL (40 mg of protein) were chromatographed on a column (1.5 × 22 cm) of Sepharose CL-4B. The samples and column were equilibrated in standard buffer containing 1 mM PMSF. Peak I of panels A and B represents fractionated LDL and tryptic LDL core particles (T-LDL), respectively. The flow rate was 40 mL/h, and 1.5-mL fractions were collected. The fractions were analyzed for protein (●) and for [^{3}H]heparin binding (○) as described under Materials and Methods. The peak I fractions were pooled; the lipoproteins were then reisolated by density-gradient ultracentrifugation and used in subsequent experiments.

The ability of LDL and T-LDL to form soluble complexes with [^{3}H]heparin is also shown in Figure 1. The amount of [^{3}H]heparin bound to LDL was constant in fractions 15–23 (4.45 μg/mg of LDL–phospholipid) but then decreased to 3.80 μg/mg of phospholipid in fraction 30. In contrast, the amount of heparin bound to T-LDL increased to 5.15 μg/mg of phospholipid in fraction 30, indicating a nonuniform surface modification of LDL by trypsin; i.e., binding of [^{3}H]heparin decreased with decreasing LDL particle size (Figure 1A) while binding increased with decreasing T-LDL particle size (Figure 1B). Thus, trypsin treatment increased the formation of soluble complexes of [^{3}H]heparin and lipoprotein. The binding of [^{3}H]heparin to the low molecular weight peptides of peak II was not determined because these peptides were not retained by the 0.022-μm filters used in the heparin-binding assay.

To examine the effects of trypsin treatment on the structure, function, and composition of LDL, the fractions corresponding to peak I (Figure 1A,B) were pooled, and the lipoproteins were reisolated by density-gradient ultracentrifugation. T-LDL had a phospholipid to protein weight ratio of 1.09 vs. 0.76 for LDL; the ratio of phospholipid to total cholesterol was not significantly different than that of LDL. The flotation densities of LDL and T-LDL were 1.027 and 1.021 g/mL (n = 2), respectively. The decrease in flotation density of T-LDL is attributed to the loss of surface protein (Figure 1B, peak II). CD analysis gave 46% α-helix, 12% β-structure, and 42% remainder for LDL as compared to 37% α-helix, 16% β-structure, and 48% remainder for T-LDL. These data indicate a loss of ordered structure in T-LDL, primarily α-helix.

The fluorescent probes 1,6-diphenylhexa-1,3,5-triene (DPH) and 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) were incorporated into LDL and T-LDL, and the temperature dependence of their fluroescence polarizations was determined (Figure 2). The fluorescent probe TMA-DPH partitions into the outer surface monolayer (Prendergast et al., 1981) whereas DPH partitions within the core and at the monolayer–core interface (Sklar et al., 1980). As shown in Figure 2, the fluorescence polarization of these probes decreases monotonically with increasing temperature. At all temperatures investigated, the polarization values for T-LDL were lower than those for LDL, indicating that T-LDL have a less rigid surface structure and, possibly, core structure than LDL.

To determine the effect of trypsin treatment on LDL function, the ability of LDL and T-LDL to inhibit the incorporation of [1-^{14}C]acetate into cellular sterols was examined. As shown in Figure 3, 0.5 μg of LDL phospholipid/mL reduced [1-^{14}C]acetate incorporation into sterols by 50%, whereas 2.5 μg of T-LDL phospholipid/mL was required to give the equivalent inhibition.

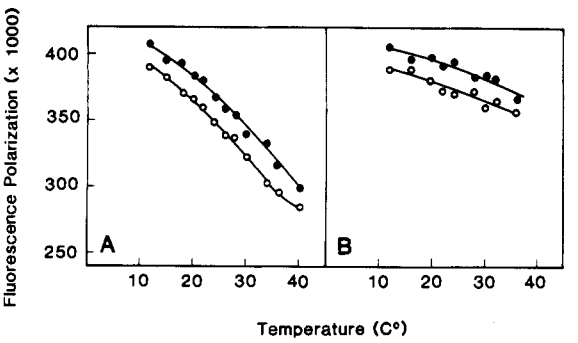

FIGURE 2: Temperature dependence of the fluorescence polarization of (A) DPH and (B) TMA-DPH incorporated into LDL (●) and T-LDL (○).

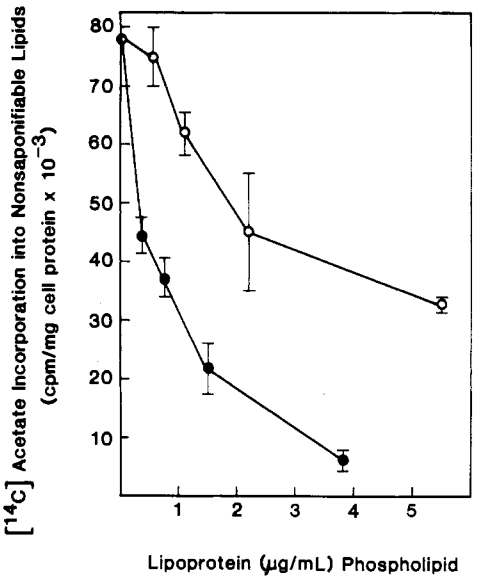

FIGURE 3: Effect of LDL (●) and T-LDL (○) on the inhibition of [1-^{14}C]acetate incorporation into nonsaponifiable lipids of normal human fibroblasts.

In contrast to the decreased interaction of T-LDL with cells, trypsin treatment did not decrease the ability of LDL to form soluble complexes with [^{3}H]heparin (Figure 1A,B). It is known that LDL also form insoluble complexes with heparin in the presence of divalent cations (Burnstein & Scholnick, 1973). To examine this interaction, a high reactive heparin was isolated by chromatography of crude heparin on LDL–AffiGel-10 in the presence of calcium ions (Cardin et al., 1984a). The calcium dependence of the formation of insoluble heparin–lipoprotein complexes was then examined (Figure 4). As the concentration of Ca^{2+} increased from 2 to 25 mM, increasing amounts of lipoprotein complexes with ^{125}I-HRH were formed. Between 5 and 25 mM Ca^{2+}, visible precipitation occurred, and ≃10% less complex precipitated as T-LDL than LDL. However, below 4 mM Ca^{2+}, the solutions remained clear. These results show that between 2 and 4 mM Ca^{2+} substantial amounts of soluble complexes were formed whereas above 5 mM Ca^{2+} precipitation occurred.

Figure 5 compares the amount of *soluble* complexes formed by LDL and T-LDL with ^{125}I-HRH in the presence of 2 mM Ca^{2+}. Between 30 and 120 μg of added phospholipid, the amounts of soluble complexes formed with LDL or T-LDL

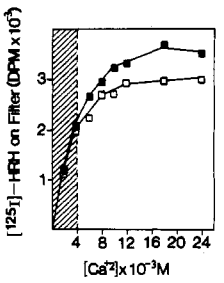

FIGURE 4: Dependence of calcium concentration on the formation of lipoprotein–heparin complexes. The shaded area denotes the region of *soluble* complex formation. The unshaded area denotes the region of *insoluble* complex formation. Each sample received 50 μg of phospholipid/mL of LDL (■) or T-LDL (□) and 2 μg of ^{125}I-HRH (2 dpm/ng).

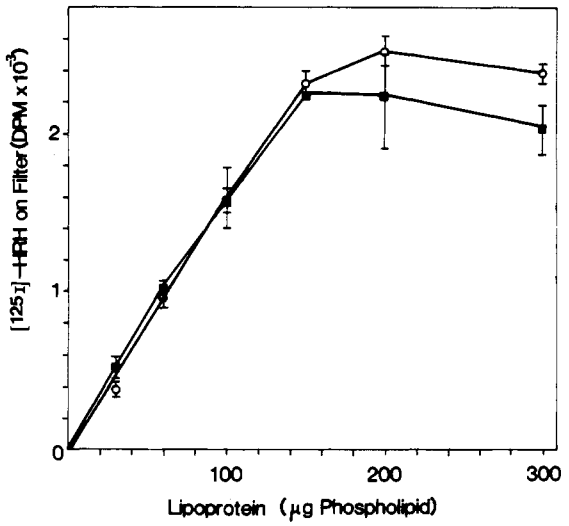

FIGURE 5: Formation of soluble lipoprotein–heparin complexes in the presence of 2 mM Ca^{2+}. In this experiment, 2 μg of ^{125}I-HRH was titrated with increasing amounts of LDL (■) and T-LDL (○) at 2 mM Ca^{2+}.

were identical. However, between 150 and 300 μg of phospholipid, ≃10% more ^{125}I-HRH was associated with soluble complexes of T-LDL compared to LDL.

In the next experiment, the effect of lipoprotein concentration on the formation of *insoluble* complexes with ^{125}I-HRH at 10 mM Ca^{2+} was examined. ^{125}I-HRH (30 μg of uronic acid) in 10 mM $CaCl_2$ was titrated with increasing amounts of LDL and T-LDL (25–150 μg of phospholipid), and the precipitates were collected on 0.45-μm filters. In these experiments, heparin was added in excess of LDL or T-LDL. Panels A and B of Figure 6 show the amount of ^{125}I-HRH and lipid, respectively, in each insoluble complex. As is shown, ≃15% less complex as ^{125}I-HRH and phospholipid was present in samples receiving T-LDL. Therefore, less T-LDL and LDL was precipitated by ^{125}I-HRH at 10 mM Ca^{2+}. Figure 6B shows that the precipitation of LDL by ^{125}I-HRH at 10 mM Ca^{2+} was quantitative. On the basis of the specific radioactivity of ^{125}I-HRH, the filtration assay yielded uronic acid/phospholipid (w/w) ratios of 0.054 ± 0.004 (n = 18) and 0.055 ± 0.005 (n = 18) for LDL and T-LDL, respectively. Moreover, in separate experiments with nonradiolabeled HRH, complexes were pelleted by centrifugation, and the amount of phospholipid and uronic acid in the complexes was determined gravimetrically (see Materials and Methods). By this method, ratios of 0.056 ± 0.004 (n = 18) and 0.068 ± 0.005 (n = 18) were obtained for LDL and T-LDL, respectively. Therefore, trypsin treatment did not alter the ratio of phospholipid to uronic acid in the insoluble complex.

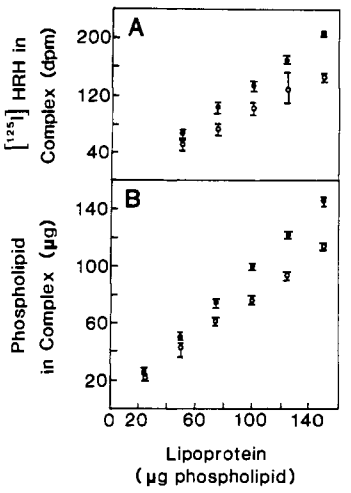

FIGURE 6: Quantitation of ^{125}I-HRH bound to insoluble complexes of LDL (●) and T-LDL (○) at 10 mM Ca^{2+}. Complexes were analyzed for uronic acid and lipoprotein mass (phospholipid). Each sample received 30 μg of ^{125}I-HRH (isotopically diluted to 34 dpm/μg of uronic acid) and increasing amounts of either LDL or T-LDL.

DISCUSSION

In the present study, LDL were extensively digested with trypsin, and the trypsin-releasable peptides were separated from the T-LDL core by gel filtration chromatography. The physical–chemical properties of T-LDL were similar to those reported previously (Chapman et al., 1978; Chen et al., 1983). We then examined the receptor- and heparin-binding properites of LDL and T-LDL under conditions of comparable lipoprotein and divalent cation concentrations. The new finding of this report is that trypsin treatment significantly reduces the binding of LDL to cell membrane receptors as shown by inhibition of incorporation of [^{14}C]acetate into cholesterol without diminishing their interaction with heparin.

Effect of Trypsin Treatment on Lipoprotein Receptor Recognition. Previous studies have shown that trypsin either diminishes or has no effect on the lipoprotein's ability to bind to cell receptors. Carew et al. (1978) reported no difference in the binding of LDL and T–LDL to the high-affinity receptors of fibroblasts in vitro. In a subsequent study, however, Chapman et al. (1984) noted decreased binding after trypsin treatment. Hahm et al. (1983) observed that trypsin treatment reduced LDL binding to the receptors in vitro. Weech et al. (1981) showed that trypsin treatment reduced the fractional catabolic rate of LDL in the guinea pig which possibly relates to diminished LDL receptor binding in vivo. The effects of trypsin treatment on LDL function are similar to those obtained with reductive methylation. For example, reductive methylation of lysine abolishes LDL binding to the receptor in vitro (Mahley et al., 1979a) and lowers their fractional catabolic rate in vivo (Mahley et al., 1979b).

The varied effects of trypsin treatment on receptor binding possibly relate to the extent of surface modification. Chapman et al. (1978) and Bernfeld and Kelly (1964) reported that trypsin treatment caused increased heterogeneity of LDL particle size and density distribution. In the present study, increased interaction with heparin is observed with decreasing particle size (Figure 1), indicating that the smaller particles are preferably degraded by trypsin. Davis et al. (1983) showed that B-74 and B-26, two proteolytic fragments of apoB (Cardin et al., 1984c), were associated with the smaller more dense LDL subfractions isolated from human vein umbilical cord blood. Furthermore, human kallikrein and thrombin, enzymes that generate fewer cleavages of apoB in LDL than does

trypsin (Cardin et al., 1984c), have a negligible effect on the binding of LDL to the fibroblast receptor in vitro (Yamamoto et al., 1985; S. Ranganathan and A. D. Cardin, unpublished observations). Thus, it is possible that more limited cleavages result in T-LDL preparations with a receptor binding activity comparable to native LDL (Carew et al., 1978), whereas more extensive degradation reduces receptor binding (Hahm et al., 1983; Chapman et al., 1984).

Effect of Trypsin Treatment on Heparin Binding. Although trypsin treatment reduced the receptor-binding capacity of LDL, the heparin-binding properties remained unaltered. These functional studies suggest that receptor binding and heparin binding are mediated through separate recognition domains. Mahley et al. (1979a) have shown that reductive methylation of 30% of the lysine residues in LDL abolished receptor binding without affecting the heparin interaction, which was assessed in both the absence and presence of the divalent cation Mn^{2+}. Scatchard (1949) analysis of the binding of monoclonal antibodies to LDL (Milne & Marcel, 1982; Tikkanen & Schonfeld, 1985) indicates a single receptor recognition domain per LDL particle. The latter study employed heparin–Mn^{2+} precipitation of LDL to quantitate the amount of antibody bound at or near the receptor recognition domain on LDL. We infer from this study that the masking of the receptor recognition site does not inhibit the lipoprotein's interaction with heparin and divalent cations. Furthermore, delipidated and denatured apoB binds heparin as determined by ligand blotting with ^{125}I-HRH (Cardin et al., 1984d) and by the ability of delipidated, water-soluble apoB (Cardin et al., 1982a) and LDL to elute from heparin–Sepharose at $\simeq$0.26 M NaCl. However, lipid-free apoB does not bind the fibroblast LDL–receptor in vitro (A. D. Cardin and S. Ranganathan, unpublished observations). Marcel et al. (1984) have shown that the expression of the apoB epitopes associated with recognition is lipid dependent. Unlike receptor recognition, lipid is not a requirement for binding of heparin to apoB.

Metabolic Implications. Bihari-Varga et al. (1982) reported that trypsinized LDL had an enhanced reactivity with an aortic glycosaminoglycan in the presence of Ca^{2+}. However, the receptor-binding properties of the T-LDL preparations were not characterized. In the absence (Figure 1) and presence (Figure 5) of 2 mM Ca^{2+}, T-LDL showed an enhanced capacity to form soluble complexes with heparin. To our knowledge, this is the first report of soluble complexes of LDL and heparin in the range of physiological Ca^{2+} concentrations. Moreover, at Ca^{2+} concentrations >5 mM, complex formation is increased, and both the LDL– and T-LDL–heparin complexes precipitate (Figure 4). The stoichiometries of the precipitated complexes were indistinguishable, but 15% less complex formed with T-LDL. The decreased precipitation is possibly a consequence of the altered interaction of Ca^{2+} with the trypsinized surface (Figure 4). This could lower the solubility product constant (K_{sp}) of the T-LDL–HRH complex relative to that of LDL. Whether proteases act in vivo to decrease the interaction of LDL with receptors and potentiate interactions with cellular and extracellular GAG is of interest as it relates to the initiation and progression of atherosclerosis. It is tempting to speculate that the interaction of soluble LDL–Ca^{2+}–GAG complexes of plasma (Nakashima et al., 1975) with calcified tissue would decrease complex solubility (increase K_{sp}), thereby promoting complex deposition in the arterial wall (Camejo et al., 1980; Hoff et al., 1974).

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Structural Changes in Membranes of Large Unilamellar Vesicles after Binding of Sodium Cholate†

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ABSTRACT: The interaction of the bile salt cholate with unilamellar vesicles was studied. At low cholate content, equilibrium binding measurements with egg yolk lecithin membranes suggest that cholate binds to the outer vesicle leaflet. At increasing concentrations, further bile salt binding to the membrane is hampered. Before the onset of membrane solubilization, diphenylhexatriene fluorescence anisotropy decreases to a shallow minimum. It then increases to the initial value in the cholate concentration range of membrane solubilization. At still higher cholate concentrations, a drop in fluorescence anisotropy indicates the transformation of mixed disk micelles into spherical micelles. Perturbation of the vesicle membranes at molar ratios of bound cholate/lecithin exceeding 0.15 leads to a transient release of oligosaccharides from intravesicular space. The cholate concentrations required to induce the release depend on the size of the entrapped sugars. Cholesterol stabilizes the membrane, whereas, in spite of enhanced membrane order, sphingomyelin destabilizes the membrane against cholate. Freeze–fracture electron microscopy and phosphorus-31 nuclear magnetic resonance (^{31}P NMR) also reflect a change in membrane structure at maximal cholate binding to the vesicles. In ^{31}P NMR spectra, superimposed on the anisotropic line typically found in phospholipid bilayers, an isotropic peak was found. This signal is most probably due to the formation of smaller vesicles after addition of cholate. The results were discussed with respect to bile salt/membrane interactions in the liver cell. It is concluded that vesicular bile salt transport in the cytoplasm is unlikely and that cholate binding is restricted to the outer leaflet of the canalicular part of the plasma membrane.

Bile acids play an important role in the absorption of dietary lipids from the intestine. De novo synthesis from cholesterol and conjugation in the liver cell are well understood. Reabsorption into the liver during enterohepatic circulation seems to be due to carrier-mediated cotransport with sodium through the sinusoidal part of the plasma membrane of the liver cell [for reviews, see Matern & Gerok (1979) and Blitzer & Boyer (1982)].

The mechanism of bile salt excretion, however, remains controversial. On the one hand, a carrier-mediated transport through the bile canalicular part of the liver cell plasma membrane cannot be excluded (Erlinger, 1981). On the other, electron microscopic studies under conditions of enhanced bile salt excretion (Boyer et al., 1978; Jones et al., 1979) suggested transport of bile salts in Golgi vesicles and exocytosis across the canalicular membrane.

For further elucidation of the mechanisms of bile salt transport in the liver cell and their interaction with the canalicular membrane, we investigated the interaction of cholate with unilamellar vesicles. The structure of lipid/bile acid mixed micelles has been studied repeatedly (Small, 1967; Mazer et al., 1976; Müller, 1981). In our opinion, unilamellar vesicles are better suited as a cellular membrane model than either mixed micelles or multibilayers when the effects of asymmetric bile acid uptake are to be studied.

Materials and Methods

Vesicle Preparation. Large unilamellar vesicles of homogeneous size were prepared by using the method of fast and controlled dialysis of mixed detergent/lipid micelles (Milsmann, 1978). Bile salts, cholesterol, lecithin from fresh egg yolk, and sphingomyelin from egg yolk were purchased from Sigma Chemical Co., St. Louis, MO.

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