Stevens, R. C., Gouaux, J. E., & Lipscomb, W. N. (1990) *Biochemistry 29*, 7691–7701.
Stevens, R. C., Reinisch, K. M., & Lipscomb, W. N. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6087–6091.
Vieira, J., & Messing, J. (1987) *Methods Enzymol. 153*, 3–11.
Weber, K. K. (1968) *Nature 218*, 1116–1119.
Wild, J. R., Loughrey-Chen, S. J., & Corder, T. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 46–50.

Role of Apolipoprotein E in Hepatic Lipase Catalyzed Hydrolysis of Phospholipid in High-Density Lipoproteins†

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ABSTRACT: We reported earlier that hepatic lipase (HL)-catalyzed hydrolysis of phospholipid monolayers is activated by apolipoprotein (apo) E [Thuren et al. (1991b) *J. Biol. Chem. 266*, 4853–4861]. On the basis of these studies, it was postulated that apoE-rich high-density lipoproteins (HDL) were preferred substrates for HL. In the present study, we tested this hypothesis, as well as further characterizing the activation of HL hydrolysis of phospholipid by apoE. The apoE-rich HDL, referred to as HDL-I, were isolated by heparin–Sepharose chromatography, and the phospholipid hydrolysis by HL was compared to an apoE-poor HDL, designated HDL-II. The hydrolysis of HDL-I phosphatidylcholine was approximately 3-fold higher than HDL-II, supporting the hypothesis that HL preferably hydrolyzes the phospholipids in apoE-rich HDL. In order to gain additional insight into the nature of the activation, we used phospholipid monolayers as model systems. Comparison of the ability of the two thrombolytic fragments of apoE (22 kDa, residues 1–191; 12 kDa, residues 192–299) revealed that only the 12-kDa fragment was capable of activating the hydrolysis of phospholipid by HL (1.75-fold). However, activation was less than with the intact protein (2.8-fold for apoE3), suggesting that the intact protein was required for full activation. The fact that the 12-kDa fragment, which represents a major lipid region of the protein, did activate HL suggests that activation occurs at the lipid–water interface. The three common isoforms of apoE differed significantly in their abilities to activate HL; apoE3, apoE2, and apoE4 activated HL 2.8-, 2.3-, and 2.0-fold, respectively, at a surface pressure of 12.5 mN/m. The order of affinity of the isoforms for PC monolayers (E4 > E2 > E3) was not the same as activation, suggesting that lipid binding per se was not the major factor distinguishing the abilities of the three isoforms to activate hydrolysis. Determination of the ratio of HL and apoE at a phospholipid interface revealed that the stoichiometry was approximately 1:1, suggesting that the activation of HL by apoE is the result of protein–protein interaction.

Hepatic lipase (EC 3.1.1.34) (HL)[1] hydrolyzes the *sn*-1 fatty acyl ester bonds of *sn*-3 phospholipids as well as the *sn*-1 (*sn*-3) ester bonds of mono-, di-, and triacylglycerols (Jackson, 1983; Kinnunen, 1984; Waite, 1987). The enzyme is located on the surface of vascular endothelium in liver where it catalyzes the hydrolysis of lipid components of plasma lipoproteins (Kinnunen, 1984; Waite, 1987). The role of HL in lipoprotein metabolism is unclear. Its broad substrate specificity and the fact that this enzyme does not absolutely require an apolipoprotein (apo) cofactor for activity have resulted in uncertainty as to what the physiological substrate(s) for this enzyme is (are). HL has been proposed to hydrolyze intermediate-density lipoprotein triacylglycerol and chylomicron remnant triacylglycerol (Dolphin, 1985; Breslow, 1988; Carlson et al., 1986; Little & Connelly, 1986; Breckenridge et al., 1982). Also it has been suggested that HL hydrolyzes HDL-II and/or apoE-rich HDL-I phospholipid (Kinnunen, 1984; Belcher et al., 1985; Jansen et al., 1980; Van Tol et al., 1980; Bamberger et al., 1985; Kuusi et al., 1979) and that the hydrolysis of HDL phospholipids by HL enhances cholesterol (ester) uptake by liver cells (Van't Hoof et al., 1981).

The effect of apolipoproteins on HL activity varies greatly depending on the assay system (Kinnunen & Ehnholm, 1976; Shinomiya, 1982; Kubo et al., 1982; Jahn et al., 1981, 1983; Landis et al., 1987). This is because the activity of HL as well as other lipolytic enzymes is greatly affected by the physicochemical state of the substrate (Waite, 1987). Using a well-controlled monolayer technique with a zero-order trough

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[1] Abbreviations: apo, apolipoprotein; apoE3-12 kDa, 12-kDa thrombolytic fragment of apoE3/E3 isoform; apoE3-22 kDa, 22-kDa thrombolytic fragment of apoE3/E3 isoform; bME, β-mercaptoethanol; diC12PE, 1,2-didodecanoylphosphatidylethanolamine; HDL, high-density lipoprotein(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; HL, hepatic lipase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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where the physicochemical state of the substrate is carefully controlled and maintained throughout HL reaction, we showed that apoE activates HL-catalyzed hydrolysis of phospholipid 3-fold at low surface pressures (Thuren et al., 1991b). The mechanism of HL activation by apoE is still unclear. It is known that apoE, as well as apoA-I, apoA-II, apoC-I, apoC-II, and apoC-III, inhibited this enzyme at high surface pressures (20 mN/m) (Thuren et al., 1991b). These previous studies were carried out using phosphatidylethanolamine (PE) monolayers as model substrates for HL since PE is the best substrate for HL. Long-chain acyl-PC monolayers are degraded by HL, but the presence of albumin in the subphase prevents the use of surface pressures below 20 mN/m. HL does not hydrolyze medium-chain saturated phosphatidylcholine (PC) monolayers, a property also shared by lipoprotein lipase and pancreatic lipase (Pieroni & Verger, 1979; Vainio et al., 1983). We have also shown using mixed micellar rabbit liver PC–Triton X-100 substrates for HL (Thuren et al., 1991a) that PC hydrolysis can be activated by apoE, which is consistent with our monolayer data. These results led us to hypothesize that phospholipid in an apoE-rich HDL-I is a preferred substrate for HL (Thuren et al., 1991b).

In the present studies, we confirm this hypothesis by studying apoE-rich and apoE-poor HDL as substrates for HL. In addition, we further characterize the activation of HL by apoE using the PE monolayer as a model substrate (Thuren et al., 1991b) by examining activation by the 22- and 12-kDa fragments of apoE, as well as by the three structural isoforms of apoE.

Experimental Procedures

Materials. Synthetic 1,2-didodecanoyl-*sn*-glycero-3-phosphoethanolamine (diC12PE), 1,2,3-tripentadecanoylglycerol, 1,2,3-trioleoylglycerol, and egg PC cholesterol, iodine, and potassium iodide were from Sigma Chemical Co. Beef heart choline plasmalogen was from Serdary Laboratories (Canada). [^{3}H]- and [^{14}C]palmitic acids were purchased from New England Nuclear. Sodium [^{125}I]iodide and *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate were purchased from Amersham Corp. (Arlington Heights, IL). *Crotalus adamanteus* phospholipase A_2 and sodium heparin were from Sigma. 1-[^{14}C]Palmitoyl- and 1-[^{3}H]palmitoyl-*sn*-glycero-3-phosphocholine were prepared from PC plasmalogen and [^{14}C]- and [^{3}H]palmitic acids using rat liver microsomes (Kucera et al., 1988). 1-[^{3}H]Palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine was prepared from the 1-[^{3}H]-palmitoyl-2-acyl-*sn*-glycero-3-phosphocholine using *Crotalus adamanteus* phospholipase A_2. Fatty acid free bovine serum albumin was from Pierce Chemicals. All other analytical grade chemicals were either from Sigma or from Fisher.

Proteins. Rat HL was purified from liver perfusate according to a modified method of Jensen and Bensadoun (1981; Waite et al., 1991). Before use in monolayer experiments, detergents were removed from the purified HL using heparin–agarose (Sigma) chromatography. HL was iodinated with sodium [^{125}I]iodide (17.4 mCi/μg; Amersham Corp.) as described earlier (Vainio et al., 1983; Kinnunen, 1977; Slotboom et al., 1978; Jackson et al., 1980). The iodinated enzyme contained 4.2×10^4 cpm/μg of protein, corresponding to 0.55 molecule of iodide incorporated per lipase molecule, and had the same specific enzymatic activity as the unlabeled enzyme. ApoE was purified from the plasma of subjects with the E2/2, E3/3, and E4/4 phenotypes as described by Rall et al. (1986). The three major apoE isoforms are designated apoE2, -E3, and -E4 and differ by cysteine-arginine interchanges at residues 112 and 158 (Zannis & Breslow, 1981; Weisgraber et al., 1981; Rall et al., 1982). The three alleles coding for these isoforms occur at a single gene locus, resulting in six common phenotypes: three homozygous (E2/2, E3/3, E4/4) an three heterozygous (E4/2, E4/3, E3/2). To study the specific effects of each isoform, we isolated apoE from the plasma of subjects with the homozygous phenotypes E2/2, E3/3, and E4/4. The 12- and 22-kDa thrombin fragments of apoE3 were prepared as described previously (Rall et al., 1986). ApoE isoforms and fragments were labeled with [^{125}I]iodine using *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate as the labeling reagent according to the method of Bolton and Hunter (1973). The iodinated apoE preparations contained $(5–15) \times 10^3$ cpm/μg.

Isolated Lipoprotein Hydrolysis Experiments. Human plasma was obtained from a single male donor (23 years old). HDL (1.063–1.15 g/mL) was isolated using flotation ultracentrifugation according to Chung et al. (1986). After dialysis against 2.5 mM Tris-HCl (pH 7.4) containing 25 mM NaCl, and HDL sample was fractionated over heparin–Sepharose according to the method of Weisgraber and Mahley (1980). The unretarded apoE-poor HDL was designated HDL-II, and the preparation eluted with 75 mM NaCl and rich in apoE was designated HDL-I. The presence of apoE was verified in the latter preparation by using SDS–PAGE electrophoresis and staining with silver. After overnight dialysis against 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, the HDL preparations were used in the hydrolysis experiments. The hydrolysis reaction mixture consisted of 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2.5 mM $CaCl_2$, 40 IU heparin, 0.3% (v/v) glycerol, and 25 mg/mL fatty acid free bovine serum albumin in a total volume of 4.14 mL. The reaction was initiated by HL (3.5 μg in 140 μL) and allowed to incubate for 3 h at 37 °C. The reaction mixtures were extracted using the procedure of Bligh and Dyer (1959), which was modified by adding 0.5 mL of acetic acid into the mixture (volume of 25 mL) to increase the recovery of lipids. Before the extraction procedure, 1-[^{3}H]palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine (1×10^6 dpm), 1-[^{14}C]palmitoyl-2-acyl-*sn*-glycero-3-phosphocholine (0.5×10^6 dpm), and 1,2,3-tripentadecanoylglycerol (10 μg) were added to the reaction mixtures as internal standards to quantitate the yields of the radioactive lipid classes. The lipid extracts were divided in half and separated using TLC. The first half was chromatographed using a phospholipid developing system of chloroform/methanol/NH_4OH/water (65:20:3:2, v/v), and the second half was chromatographed using a neutral lipid elution system of hexane/isopropyl ether/acetic acid (60:40:3, v/v). The regions corresponding to PE, PC, sphingomyelin, and lyso-PC were scraped and eluted, and the lipid phosphorus was quantitated (Chalvardjian & Rudnicki, 1970). PC and lyso-PC samples eluted from silica were counted for ^{3}H and ^{14}C to correct the results for recoveries. The regions corresponding to cholesteryl ester, triacylglycerol, and unesterified cholesterol were scraped and eluted. Cholesteryl ester and unesterified cholesterol were determined after methylation using gas–liquid chromatography and an Econo-Cap column SE-30 (Alltech Associates Inc., Deerfield, IL) according to manufacturers' instructions. Triacylglycerol was determined based on fatty acid content, which was measured using gas–liquid chromatography (OV-351 column, J&W Scientific, Folsom, CA) after esterified fatty acids were converted into fatty acyl methyl esters (Kuksis, 1978). An internal standard of pentadecanoic acid was used to determine the triacylglycerol recoveries. Isolated lipoprotein hydrolysis experiments were done in triplicate, and results in Table II are means plus

standard deviations of these experiments. In addition, these results were verified using plasma samples from six individuals in separate experiments.

Monolayer Experiments. The surface barostat method was used to determine the rate of hydrolysis of lipid monolayers at a constant surface pressure (Thuren et al., 1991b; Verger & deHaas, 1973, 1976) using a KSV LB5000 monolayer apparatus. Enzyme kinetics were followed in a key-type Verger–deHaas zero-order trough (Verger & deHaas, 1976; Lairon et al., 1980) as described earlier (Thuren et al., 1991b). The trough consisted of a reaction compartment (total volume 35 mL, total surface 23.2 cm^2), a compartment for the platinum plate, and a film reservoir (20 × 155 mm). The compartments were connected by shallow, narrow channels on sandblasted glass slides. The aqueous subphase consisted of 20 mM Tris-HCl, pH 8.4, 0.5 M NaCl, and 5 mM $CaCl_2$ prepared with deionized and filtered water (Pure-Flow Inc., Mebane, NC). DiC12PE monolayers were spread from chloroform. The apoE isoforms and fragments were injected beneath the monolayer 5 min before the injection of the enzyme to initiate the reaction. The constant surface pressure used in these studies was 12.5 mN/m. After the reaction, the lipid films were recovered and counted for radioactivity according to the method of Rietsch et al. (1977).

The adsorption of apoE isoforms to lipid monolayers was studied using a Teflon well that had a total surface area of 12 cm^2 and contained 17 mL of 20 mM Tris-HCl buffer (pH 7.4), 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.05% β-mercaptoethanol (bME) at room temperature. A lipid monolayer was spread to indicate initial surface pressures at a clean air–water interface from a chloroform solution using a 100-μL Hamilton syringe. The lipid film was allowed to equilibrate 15 min before an iodinated apoE sample was injected beneath the lipid monolayer through an inlet port without disturbing the lipid film. All apoE preparations were injected from 0.1 M NH_4HCO_3 (pH 7.8) containing 0.5% bME to eliminate disulfide-linked multimers. The injection of 0.1 M NH_4HCO_3 containing 1.0% bME did not result in any changes in the surface pressure of the lipid monolayer. The subphase was constantly stirred during the adsorption experiments at 250 rpm using magnetic stirring bars (5 mm × 1 mm). The adsorption of apoprotein samples to the interface was monitored by measuring the increase in the surface pressure of the film versus time. After the surface pressure change was stabilized, usually 60 min after the injection of an apoprotein sample into the subphase, the film was collected and the radioactivity determined according to the method of Rietsch et al. (1977). These experiments were done in duplicate. Data from all monolayer experiments were collected into a personal computer and analyzed with KSV LB5000 software (KSV-Instruments, Stamford, CT). The error in determining the amount of surface-adsorbed protein was approximately ±10%. The surface pressure was measured by using the Wilhelmy plate technique employing a roughened platinum plate and a KSV LB5000 monolayer apparatus (Thuren et al., 1991b). The reproducibility of surface pressure measurements using this technique was ±0.5 mN/m.

Results

Hydrolysis of ApoE-Rich and ApoE-Poor HDL by HL. To test our hypothesis that apoE-rich HDL (HDL-I) is a preferred substrate for HL (Thuren et al., 1991b), we compared the hydrolysis of HDL-I and HDL-II by HL. The percentage compositions of apoE-poor (HDL-II) and apoE-rich (HDL-I) HDL are shown in Table I. The protein content of HDL-I is lower than HDL-II, whereas the phospholipid and unesterified cholesterol content is higher. The two HDL subclasses were characterized by light scattering. The average size of HDL-I, expressed as the Stokes radius, was 13.6 nm, whereas that of HDL-II was 10.8 nm. These values are in good agreement with values obtained from negative-staining electron microscopy, 13.5 ± 1.0 and 10.6 ± 1.0 nm for HDL-I and HDL-II, respectively (data not shown).

Table I: Composition of Human ApoE-Rich and Apo-Poor HDL[a]

	mean % (by wt)				
	CE	TG	FC	PL	protein
HDL-I	6.2	3.2	5.8	37.0	40.5
HDL-II	6.4	4.0	3.8	31.6	54.3

[a] HDL subclasses were isolated using heparin–Sepharose chromatography. HDL-I, apoE-rich high-density lipoprotein; HDL-II, apoE-poor high-density lipoprotein; CE, cholesterol ester; TG, triacylglycerol; FC, free cholesterol; PL, phospholipid.

Table II: Hydrolysis of ApoE-Rich and Apo-Poor HDL[a]

	HDL-I (nmol/incubation)		HDL-II (nmol/incubation)	
	−HL	+HL	−HL	+HL
LPC	136.3 ± 7.8	351.0 ± 21	73.8 ± 4.3	141.9 ± 14.2
Δ	+214.7		+68.1	
SPM	84.5 ± 5.4	98.3 ± 6.5	85.6 ± 7.0	79.4 ± 11.5
Δ	+13.8		−6.2	
PC	689.0 ± 13.5	490.0 ± 5.1	640.5 ± 30.9	570.7 ± 8.8
Δ	−199.0		−69.8	
PE	16.2 ± 3.3	6.5 ± 2.0	17.6 ± 7.6	9.7 ± 4.4
Δ	−9.7		−7.9	
CE	178.2 ± 28.6	188.9 ± 11.8	194.6 ± 11.8	190.2 ± 9.2
FC	281.7 ± 40.1	314.2 ± 28.9	193.0 ± 12.1	205.2 ± 7.5
TG	68.2 ± 2.9	45.4 ± 1.0	78.2 ± 6.7	60.4 ± 2.8
Δ	−22.8		−17.8	

[a] HDL subclasses were isolated using heparin–Sepharose chromatography. Incubations with 3.5 μg of purified HL were performed as described under Experimental Procedures. HDL-I, apoE-rich high-density lipoprotein; HDL-II, apoE-poor high-density lipoprotein; LPC, lysophosphatidylcholine; SPM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CE, cholesterol ester; FC, free cholesterol; TG, triacylglycerol; Δ, difference in nanomoles of indicated lipid between incubations carried out with the enzyme and without the enzyme.

Approximately equal amounts of phospholipids from both HDL subclasses were hydrolyzed by incubation with HL. The hydrolysis of PC from HDL-I produced approximately 3-fold more lyso-PC (215 nmol) than was obtained from HDL-II (68 nmol, Table II). The decrease in PC (199 and 70 nmol) corresponded well to the increase in lyso-PC for HDL-I and HDL-II, respectively. Both HDL-I and HDL-II PE were also hydrolyzed; the decreases were 9.7 and 7.9 nmol, respectively (Table II). The triacylglycerol content of HDL-I and HDL-II decreased 22.8 and 17.8 nmol, respectively (Table II). As expected, HL did not hydrolyze sphingomyelin significantly. Both the cholesterol ester and unesterified cholesterol contents of HDL-I and HDL-II were not affected by HL (Table II).

Activation of HL by ApoE Fragments and Isoforms. Next we studied the ability of apoE fragments and isoforms to activate HL using diC12PE monolayers as substrates for HL. As apoE has been demonstrated to contain two independently folded domains, that can be modeled by thrombolytic fragments of the protein (Wetterau et al., 1988), it was of interest to determine if the ability to activate HL was associated with one of the domains. The 12-kDa fragment of isoform E3 activated HL 1.75-fold, whereas the 22-kDa fragment of isoform E3 was unable to activate HL at the surface pressure of 12.5 mN/m (Table III). Thus, it appears that intact apoE is required for maximum activation of HL. Because intact apoE inhibited HL at higher surface pressures (Thuren et al.,

Table III: Activation of HL-Catalyzed Hydrolysis of Phospholipid Monolayers by ApoE[a]

surface pressure (mN/m)	addition	activation factor
12.5	none	1.00 ± 0.02
	E2	2.32 ± 0.06
	E3	2.64 ± 0.11
	E4	2.04 ± 0.14
	E3-12-kDa fragment	1.75 ± 0.05
	E3-22-kDa fragment	1.12 ± 0.06
	E3 in NH_4HCO_3	1.78 ± 0.11
	E3 in bME	2.82 ± 0.12
	E3 without NaCl	0
25	E3-12-kDa fragment	0.13 ± 0.10
	E3-22-kDa fragment	0.95 ± 0.03

[a] Monolayer measurements were performed as described under Experimental Procedures. Activation factors were calculated by dividing the HL activity (in the presence of the indicated addition) by the activity in the absence of the additive. HL (0.5 μg) was used in each experiment; 1 μg of apoE isoforms, 0.35 μg of 12-kDa fragment, and 0.6 μg of 22-kDa fragment. Apo was injected beneath a diC12PE monolayer from a 0.5% bME in NH_4HCO_3 solution (final subphase concentration 0.002%) if not otherwise indicated, 5 min before the addition of the enzyme. bME was omitted from the injection buffer and from the subphase in the experiment "E3 in NH_4HCO_3". In experiment "E3 in bME", the subphase contained 0.05% bME. In experiment "E3 without NaCl," 0.5 M NaCl was omitted from the subphase buffer of 20 mM Tris-HCl, pH 8.4, 0.5 M NaCl, and 5 mM $CaCl_2$. Student's t-test gave $p < 0.001$ for activation factors of apoE2, apoE3, and apoE4 when compared to no addition ($n = 8$ in each case). $p < 0.001$ was obtained for the activation factors of apoE2 and apoE4 when compared to that of apoE3 and $p < 0.001$ for comparison of the activation factor of apoE4 to that of apoE2 ($n = 8$ in each case).

1991b), we examined the effects of the thrombolytic fragments of apoE on HL at higher surface pressures. At the surface pressure of 25 mN/m, only the 12-kDa fragment inhibited HL hydrolysis of the diC12PE monolayer (87% inhibition) (Table III).

Since the genetic polymorphism of apoE is well-known (Zannis & Breslow, 1981; Weisgraber et al., 1981; Rall et al., 1982) and these structural isoforms are known to have an impact on function, it was of interest to determine if they differed in their effects on HL activation. As shown in Table III, apoE3 activated HL 2.64-fold when it was injected from a 0.5% bME solution (final concentration of 0.002% bME in the subphase), and these was a 2.82-fold activation in the presence of 0.05% bME in the subphase. When apoE3 was injected beneath the monolayer in 0.1 M NH_4HCO_3 in the absence of bME, it only activated HL 1.78-fold (Table III). ApoE2 activated HL 2.32-fold and apoE4 2.04-fold, both values being significantly lower ($p < 0.001$) than that for apoE3 (Table III). In earlier studies, we showed that HL did not hydrolyze diC12PE monolayers in the absence of NaCl in the subphase and that HL exhibited maximal activity in the presence of 0.5 M NaCl (Thuren et al., 1991b). In a study to test if apoE could substitute for NaCl, we found that apoE3

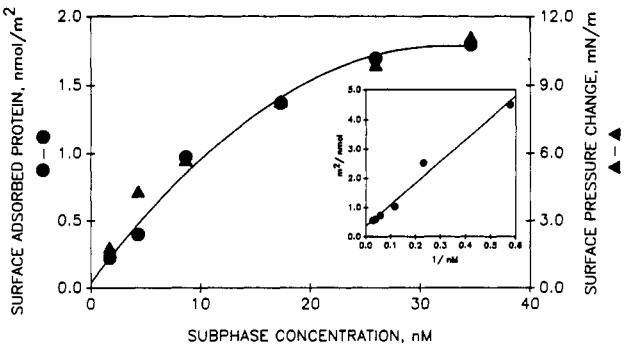

FIGURE 1: Binding of apoE2 to egg PC monolayers. The change in surface pressure (filled triangles) and the surface concentration of adsorbed ^{125}I-labeled apoE (filled circles) were measured as a function of apoE concentration in the subphase. The surface pressure change and surface concentration of apoE2 were measured 45 min after injection of the indicated amount of protein beneath a monolayer of egg PC. The initial surface pressure was 15 mN/m. The insert represents the double-reciprocal plot of the surface concentration of apoE2 as a function of subphase concentration. The dissociation constants (K_d) in Table II were determined from similar double-reciprocal plots. The x intercept of a linear least-squares method fitted regression line in the double-reciprocal plot was taken as $1/K_d$ and the y intercept as the maximal surface concentration of apoE.

did not induce hydrolysis of the diC12PE monolayer in the absence of NaCl (Table III). All apoE isoforms tested completely inhibited HL at a high surface pressure of 25 mN/m (data not shown).

Absorption of ApoE Isoforms to Lipid Monolayers. Next we studied the adsorption of apoE isoforms to egg PC monolayers to determine if the apoE absorption was related to the different abilities to apoE isoforms to activate HL. First, the critical surface pressure for penetration of apoE into egg PC monolayers was determined from the change in the surface pressure after addition of apoE as a function of the initial surface pressure (Macritchie, 1978). When the surface pressure change was extrapolated to zero, the value of 27.5 mN/m for the initial surface pressure for apo E2 penetration was obtained. The critical surface pressures for penetration of the apoE isoforms did not significantly differ from each other (mean = 29.0 ± 1.5 mN/m) (Table IV).

To determine the dissociation constant, K_d, for the adsorption of the apoE isoforms, we injected different amounts of ^{125}I-labeled isoforms or fragments beneath the monolayers at a surface pressure of 15 mN/m. The K_d values were determined from double-reciprocal plots of the subphase apoE concentration vs the absorbed apoE amount (surface density). The surface density of apoE2 in the egg PC monolayer and the corresponding surface pressure changes as a function of the subphase concentration of apoE2 are shown in Figure 1. These results show that the apoE2 adsorption into an egg PC film was saturable and that the saturation was achieved at a

Table IV: Adsorption of ApoE Isoforms into Monolayers[a]

apoE isoform	monolayer	K_d (nM)	max affinity (molecules of apoE/ 1000 PC molecules)	critical surface pressure (mN/m)
E2/E2	egg PC	20.6 ± 2.9	1.3 (2.8 nM)	27.5
E3/E3	egg PC	30.1 ± 3.6	1.4 (3.0 nM)	29.0
E4/E4	egg PC	15.7 ± 2.4	1.9 (3.9 nM)	30.4
E3-12 kDa	egg PC	33.0 ± 5.5	2.7 (5.8 nM)	27.2
E3-22 kDa	egg PC	77.9 ± 10.0	0.2 (0.5 nM)	ND

[a] K_d experiments were conducted at 15 mN/m. K_d values were determined from double-reciprocal plots similar to the insert in Figure 2. The critical surface pressure was determined as described in Figure 1. Egg PC, egg phosphatidylcholine; ND, not determined. K_d was defined to be the subphase concentration of apoE needed to achieve half of the maximal adsorption level. It was determined from the double-reciprocal plots as shown in the insert of Figure 1.

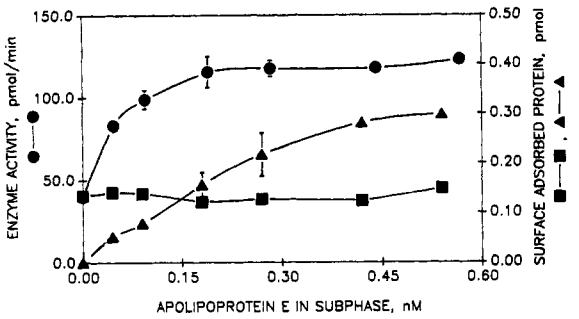

FIGURE 2: Activation of HL by apoE. HL hydrolysis of diC12PE monolayers (circles), amount of HL adsorbed to the lipid film (squares), and amount of apoE absorbed to the lipid film (triangles) were determined as a function of apoE concentration in the subphase. The enzyme activity represents the average of duplicate experiments performed in triplicate using unlabeled HL and ^{125}I-labeled apoE as well as ^{125}I-labeled HL and unlabeled apoE. The binding experiments represent the average of three experiments. The studies were performed at a surface pressure of 12.5 mN/m, and 0.41 μg of HL was injected into the subphase to initiate the reaction. See Experimental Procedures for details.

subphase concentration of approximately 30 nM (Figure 1). The double-reciprocal plot of these data is presented as an insert in Figure 1. These plots are representative of the plots obtained for the other isoforms (data not shown). The K_d values for the apoE isoforms and 12- and 22-kDa fragments are given in Table IV.

The K_ds of apoE binding to egg PC monolayers were increased in the order E4 < E2 < E3 (15.7, 20.6, and 30.1 nM, respectively (Table IV). The K_d for the 12-kDa fragment was higher (33.0 nM) than the K_d for apoE2 and apoE4 but did not differ significantly from the K_d of apoE3. In contrast, the 22-kDa fragment had an approximately 2-fold higher K_d for binding to egg PC monolayers (77.9 nM) (Table IV) than the 12-kDa fragment. The maximal affinity for apoE2, apoE3, and apoE4 was between 1 and 2 molecules of apoE/1000 molecules of egg PC (1.3, 1.4, and 1.9 for apoE2, apoE3, and apoE4, respectively). The maximal affinity of apoE3-12-kDa fragment was approximately 3 molecules/1000 molecules of PC, and that of apoE3-22-kDa fragment was 0.2.

We reported earlier that hydrolysis of PE monolayers by HL at low surface pressures can be activated 3-fold by apoE (Thuren et al., 1991b). In this study, we showed that apoE-rich HDL is hydrolyzed at a faster rate than apoE-poor HDL. Next we attempted to gain insight into the mechanism of HL activation by apoE by establishing the stoichiometry of HL and apoE at the lowest subphase concentration of apoE that resulted in maximal HL activation. Figure 2 shows the results from two types of experiments in which we studied the activation of unlabeled HL by ^{125}I-labeled apoE3 and the activation of ^{125}I-labeled HL by unlabeled apoE3. In these experiments, we measured the enzyme activity and at the end of the experiments measured the amount of protein adsorbed to the lipid film under conditions optimal for HL-catalyzed hydrolysis of phospholipid monolayers. ApoE maximally activated HL-catalyzed hydrolysis 3-fold at a surface pressure of 12.5 mN/m (Figure 2). Further, these results show that at the lowest subphase concentration of apoE (0.189 nM) that resulted in the maximal activation, 0.153 ± 0.016 pmol of labeled apoE3 was adsorbed to the diC12PE monolayer (filled triangles). The amount of HL adsorbed to the same monolayer under identical conditions was 0.138 ± 0.010 pmol (filled squares). These results indicate an approximate ratio of 1:1.1 between the lipid-adsorbed apoE and HL. At higher apoE concentrations (up to 0.6 nM), 0.3 pmol of apoE was absorbed to the film, although this did not lead to further activation of HL.

Discussion

We suggested previously that the catalytic activity of HL is regulated by both the physicochemical state (lipid packing and surface pressure) of the substrate and the apoprotein composition of lipoprotein particles (Thuren et al., 1991b). Specifically, we showed that apoE activates HL-catalyzed phospholipid hydrolysis and postulated that apoE-rich HDL phospholipid is a preferred substrate for HL. Herein we use natural substrates, apoE-rich HDL-I and apoE-poor HDL-II, to test this hypothesis. The HDL-I particles were larger and contained more lipid and less apoprotein than HDL-II, in agreement with earlier reports (Weisgraber & Mahley, 1980). Our results clearly show that HDL-I phospholipids and triacylglycerol were hydrolyzed at a higher rate than those of HDL-II. The approximately 3-fold higher rate observed for the hydrolysis of apoE-rich HDL-I PC is almost identical to the activation factor observed for apoE in monolayer studies. Lecithin–cholesterol acyltransferase is unlikely to have contributed to these results because we did not find an increase in esterified cholesterol and a corresponding decrease in free cholesterol upon incubation of the HDL (Dobiasova, 1983). The approximately 50% higher protein concentration in HDL-II incubation did not result in an inhibition of HL, since the hydrolysis was linear over a wide range of both HDL-I and HDL-II concentrations. Also, the hydrolysis of PE films (Figure 2; Thuren et al., 1991b) and PC in mixed micelles (Thuren et al., 1991a) was not decreased by an excess of apoprotein.

Although we did not observe a clear activation of triacylglycerol hydrolysis by apoE in monolayers, we did observe a 1.6-fold activation of HL-catalyzed triacylglycerol hydrolysis when using Triton X-100–triolein particles (Thuren et al., 1991a). Our results comparing HDL-I vs HDL-II triacylglycerol hydrolysis support these findings. When compared on a percentage basis, nearly 50% more triacylglycerol in HDL-I was degraded when compared with that of HDL-II (34% vs 23% hydrolyzed) (Table II). Azema et al. (1990) demonstrated that HDL triacylglycerol and PE were hydrolyzed at relatively higher rates than PC when their low concentration was considered. In this study, the relative extent of PE and triacylglycerol hydrolysis was similar to or greater than that of PC. However, in absolute terms, more PC than PE or triacylglycerol was hydrolyzed by HL. The specific activity of HL for HDL-I PC was approximately $^1/_{20}$th of that for PC–Triton X-100 mixed micelles (Kucera et al., 1988). The specific activity of HL for lipoprotein substrates reported here was in the same range as that reported by Belcher et al. (1985).

At present, we have evidence using three different substrate systems, phospholipid monolayers, phospholipid–Triton X-100 mixed micelles, and isolated HDL particles, that HL-catalyzed hydrolysis of PC and PE can be activated approximately 3-fold by apoE (Thuren et al., 1991a,b). The results we initially observed using lipid monolayers as substrates for HL are identical to those observed using naturally occurring lipoprotein particles. These observations suggest that a phospholipid monolayer represents a reasonable model for the surface lipid film of lipoproteins (Verger & deHaas, 1973; Jackson et al., 1976). In addition, the results of the present studies confirm our hypothesis that apoE-rich HDL phospholipids are preferred substrates for HL (Thuren et al., 1991b). This further implies that the lateral surface pressure of HDL particles at the level of the fatty acyl ester bonds is below 15 mN/m,

because at higher lateral surface pressures apoE and other apo inhibited HL (Thuren et al., 1991b). Related to this conclusion, porcine pancreatic phospholipase A_2 is incapable of hydrolyzing PC monolayers above a surface pressure of 16 mN/m (Demel et al., 1975). Because this enzyme also hydrolyzes HDL particles, it is reasonable to speculate that the lateral surface pressure of HDL at the level of fatty acyl ester bonds is lower than 16 mN/m (Camejo, 1969; Swaney & Orishimo, 1988).

Having established the suitability of the monolayer system as a model for the apoE activation of phospholipid hydrolysis by HL, we sought to gain further insight into the mechanism of activation. The experiments using thrombin fragments of apoE to activate HL suggest that the carboxyl-terminal portion of apoE contains elements required for activation whereas the amino-terminal end containing the receptor binding region (Wetterau et al., 1988; Aggerbeck et al., 1988; Innerarity et al., 1983) was not sufficient alone to activate HL. However, the fact that the activation factor for the 12-kDa fragment was 66% of that for the intact apoE3 suggests that the intact apoE is required for the full activation process. The requirement of the intact protein could be due to domain interaction between the amino- and carboxyl-terminal domains of apoE. There are other examples of domain interaction with apoE. The carboxyl-terminal domain has been shown to affect the receptor binding function of apoE that is contained in the amino-terminal domain (Innerarity et al., 1984; Wardell et al., 1989), and the amino-terminal domain has been shown to influence lipid binding, that is mainly determined by the carboxyl-terminal domain (Weisgraber, 1990).

As apoE is known to be polymorphic, with the three common isoforms arising from cysteine–arginine interchanges at residues 112 and 158 in the 22-kDa region (Zannis & Breslow, 1981; Weisgraber et al., 1981; Rall et al., 1982) and that this polymorphism is known to affect function, it was of interest to study the ability of the isoforms to activate HL. The results clearly indicate that apoE4 activated HL less than apoE2 and apoE3. This suggests either that the presence of arginine at position 112 changed the tertiary structure of apoE4 or that cysteine-112 was important for the activation. In addition, the substitution of cysteine for arginine at position 158 also results in a reduced ability to activate HL. This result is consistent with a previous study in which the addition of apoE3 but not apoE2 was shown to facilitate the hydrolysis of B-very-low-density lipoprotein by HL to "low-density lipoprotein-size" particles (Ehnholm et al., 1984).

To see if the differences in the ability of apoE isoforms to activate HL were due to differences in lipid binding of these isoforms, we measured the affinities of the apoE isoforms and the 22- and 12-kDa fragments to PC lipid monolayers. The order of dissociation constants, K_d, of the isoforms to PC monolayers (E4 < E2 < E3) was not the same order of activation of HL phospholipid hydrolysis (E3 > E2 > E4). These results suggest that the lipid binding affinity of these apoE preparations is not the major factor in the different abilities of apoE isoforms to activate HL. This is also consistent with the results of the 12-kDa fragment. Although this fragment had the highest affinity for PC monolayers, it had the lowest activity. Because of the possible differences between the films of egg PC used in the binding studies and diC12PE used in the hydrolysis studies, our conclusions concerning the effects of apoE are only tentative. However, in our preliminary studies, the binding of apoE3 to diC12PE films was only 25% lower than to egg PC films (data not shown) which indicates that there are no large differences in the binding of apoE3 into these two lipid monolayers. We do not use the term "binding sites" in the more conventional sense but simply use the term to express maximal apoprotein binding per unit area of film. The 12-kDa fragment had maximally approximately 6 "binding sites"/1000 PC molecules, consistent with its smaller size when compared with the full-length apoE. The low maximal "binding site" number for the 22-kDa fragment may result from a loose association with the film such that true binding does not occur.

On the basis of our earlier results on the activation of HL by apoE, we hypothesized that the activation occurred at the lipid–water interface and that it involved protein–protein interactions between HL and apoE (Thuren et al., 1991b). The results obtained in this study indicate that the stoichiometry between HL and apoE at the phospholipid film was approximately 1:1 at the lowest subphase concentration of apoE that gave full activation of HL. This result, together with the fact that different amounts of apoE in the lipid film did not change the amount of HL absorbed to the lipid film, suggests that the mechanism of HL activation by apoE involves protein–protein interaction at the lipid film. ApoE is known to self-associate primarily in the form of tetramers (Yokoyama et al., 1985; Aggerbeck et al., 1988; Weisgraber, 1990); the cysteines can also undergo oxidation to form disulfide-linked structures (Weisgraber & Shinto, 1991). The finding that apoE3 injected from a bME solution activated HL better than in the absence of bME suggests that the formation of disulfide-linked structures alters the interaction of apoE with HL or the binding of apoE to lipid films. At the present time, we do not known the aggregation state of apoE in the lipid films.

The fact that the HL-activating 12-kDa fragment contains the major lipid binding region of apoE (Wetterau et al., 1988; Aggerbeck et al., 1988) suggests that the activation occurs most likely at the lipid film and not in the aqueous subphase prior to the adsorption of HL and apoE to the lipid film. However, the mechanism of activation of apoE and the 12-kDa fragment does not involve increased HL binding to the film. Conversely, at surface pressures higher than 18 mN/m, apoE reduces HL–film interaction (Thuren et al., 1991b). The inhibition of HL by the 12-kDa fragment at higher surface pressures also suggests that this phenomenon occurs at the lipid film. In this case, apoE as well as the 12-kDa fragment bound to lipid film would inhibit the adsorption of HL into a phospholipid film at a higher surface pressure, i.e., when the lipid molecules are tightly packed. The present results, therefore, suggest that HL activation by apoE involves protein–protein interaction at the lipid film. The work to further elucidate the mechanism of apoE activation of HL-catalyzed phospholipid hydrolysis is in progress in our laboratory.

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REFERENCES

Aggerbeck, L. P., Wetterau, J. R., Weisgraber, K. H., Wu, C.-S. C., & Lindgren, F. T. (1988) *J. Biol. Chem. 263*, 6249–6258.

Azema, C., Marques-Vidal, P., Lespine, A., Simard, G., Chap, H., & Perret, B. (1990) *Biochim. Biophys. Acta 1046*, 73–80.

Bamberger, M., Glick, J. M., & Rothblat, G. H. (1983) *J. Lipid Res. 24*, 869–876.

Bamberger, M., Lund-Katz, S., Phillips, M. C., & Rothblat, G. H. (1985) *Biochemistry 24*, 3693–3701.
Belcher, J. D., Sisson, P., & Waite, M. (1985) *Biochem. J. 229*, 343–351.
Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.
Bolton, A. E., & Hunter, W. M. (1973) *Biochem. J. 133*, 529–539.
Breckenridge, W. C., Little, J. A., Alaupovic, P., Wang, C. S., Kuksis, A., Kakis, G., Lindgren, F., & Gardiner, G. (1982) *Atherosclerosis 45*, 161–179.
Breslow, J. L. (1988) *Physiol. Rev. 68*, 85–132.
Camejo, G. (1969) *Biochim. Biophys. Acta 175*, 290–300.
Cardin, A. D., Witt, K. R., & Jackson, R. J. (1984) *Anal. Biochem. 137*, 368–373.
Carlson, L. A., & Holmquist, L., & Nilsson-Ehle, P. (1986) *Acta Med. Scand. 219*, 435–447.
Chalvardjian, A., & Rudnicki, E. (1970) *Anal. Biochem. 36*, 225–232.
Chung, H. B., Segrest, J. P., Ray, M. J., Brunzell, J. D., Hokanson, J. E., Krauss, R. M., Beaudrie, K., & Cone, J. T. (1986) *Methods Enzymol. 128*, 181–209.
Demel, R. A., Guertz van Kessel, W. S. M., Zwaal, R. F. A., Roelofsen, B., & van Deenen, L. L. M. (1975) *Biochim. Biophys. Acta 406*, 97–107.
Dobiasova, M. (1983) *Adv. Lipid Res. 20*, 107–194.
Dolphin, P. J. (1985) *Can. J. Biochem. Cell Biol. 63*, 850–869.
Ehnholm, C., Mahley, R. W., Chappell, D. A., Weisgraber, K. H., Ludwig, E., & Witztum, J. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5566–5570.
Innerarity, T. L., Friedlander, E. J., Rall, S. C., Jr., Weisgraber, K. H., & Mahley, R. W. (1983) *J. Biol. Chem. 258*, 12341–12347.
Innerarity, T. L., Weisgraber, K. H., Arnold, K. S., Rall, S. C., Jr., & Mahley, R. W. (1984) *J. Biol. Chem. 259*, 7261–7267.
Jackson, R. L. (1983) *Enzymes (3rd Ed.) 16*, 141–181.
Jackson, R. L., Morrisett, J. D., & Gotto, A. M., Jr. (1976) *Physiol. Rev. 56*, 259–316.
Jackson, R. L., Pattus, F., & deHaas, G. H. (1980) *Biochemistry 19*, 373–378.
Jahn, C. E., Osborne, J. C., Jr., Schaefer, E. J., & Brewer, B. H., Jr. (1981) *FEBS Lett. 131*, 366–368.
Jahn, C. E., Osborne, J. C., Jr., Schaefer, E. J., & Brewer, B. H., Jr. (1983) *Eur. J. Biochem. 131*, 25–29.
Jansen, H., van Tol, A., & Hulsmann, W. L. (1980) *Biochem. Biophys. Res. Commun. 92*, 53–59.
Jensen, G. L., & Bensadoun, A. (1981) *Anal. Biochem. 113*, 246–252.
Kinnunen, P. K. J. (1977) *Med. Biol. 55*, 187–191.
Kinnunen, P. K. J. (1984) in *Lipases* (Borgstrom, B., & Brockman, H. L., Eds.) pp 307–328, Elsevier Science Publishers B. V., Amsterdam, The Netherlands.
Kinnunen, P. K. J., & Enholm, C. (1976) *FEBS Lett. 65*, 354–357.
Kubo, M., Matsuzawa, Y., Yokoyama, S., Tajima, S., Ishikawa, K., Yamamoto, A., & Tarui, S. (1982) *J. Biochem. 92*, 865–870.
Kucera, G. L., Sisson, P., Thomas, M. J., & Waite, M. (1988) *J. Biol. Chem. 263*, 1920–1928.
Kuksis, A. (1978) *Handb. Lipid Res. 1*, 1–76.
Kuusi, T., Kinnunen, P. K. J., & Nikkila, E. A. (1979) *FEBS Lett. 104*, 384–388.
Lairon, D., Charbonnier-Augeire, M., Nalbone, J., Leonardi, J., Hauton, J. C., Pieroni, G., Ferrato, F., & Verger, R. (1980) *Biochim. Biophys. Acta 618*, 106–118.
Landis, B. A., Rotolo, F. S., Meyers, W. C., Clark, A. B., & Quarfordt, S. H. (1987) *Am. J. Physiol. 252*, G805–G810.
Little, J. A., & Connelly, P. W. (1986) *Adv. Exp. Med. Biol. 210*, 253–260.
Macritchie, F. (1978) *Adv. Protein Chem. 32*, 283–326.
Mahley, R. W., Weisgraber, K. H., & Innerarity, T. L. (1979) *Biochim. Biophys. Acta 575*, 81–89.
Pieroni, G., & Verger, R. (1979) *J. Biol. Chem. 254*, 10090–10094.
Rall, S. C., Jr., Weisgraber, K. H., & Mahley, R. W. (1982) *J. Biol. Chem. 257*, 4171–4178.
Rall, S. C., Jr., Weisgraber, K. H., & Mahley, R. W. (1986) *Methods Enzymol. 128*, 273–287.
Rietsch, J., Pattus, F., Desnuelle, P., & Verger, R. (1977) *J. Biol. Chem. 252*, 4313–4318.
Shelburne, F. A., & Quarfordt, S. H. (1977) *J. Clin. Invest. 60*, 944–950.
Shinomiya, M., Sasaki, N., Barnhart, R. L., Shirai, K., & Jackson, R. L. (1982) *Biochim. Biophys. Acta 713*, 292–299.
Slotboom, A. J., Verheij, H. M., Puijk, W. C., Dedieu, A. G. R., & deHaas, G. H. (1978) *FEBS Lett. 92*, 361–364.
Swaney, J. B., & Orishimo, M. W. (1988) *Lipids 23*, 214–219.
Thuren, T., Sisson, P., & Waite, M. (1991a) *Biochim. Biophys. Acta 1083*, 217–220.
Thuren, T., Wilcox, R. W., Sisson, P., & Waite, M. (1991b) *J. Biol. Chem. 266*, 4853–4861.
Vainio, P., Virtanen, J. A., Kinnunen, P. K. J., Gotto, A. M., Jr., Sparrow, J. T., Pattus, F., Bougis, P., & Verger, R. (1983) *J. Biol. Chem. 258*, 5477–5482.
Van Tol, A., van Gent, T., & Jansen, H. (1980) *Biochem. Biophys. Res. Commun. 94*, 101–108.
Van't Hoof, F. M., van Gent, T., & van Tol, A. (1981) *Biochem. J. 196*, 877–885.
Verger, R., & deHaas, G. H. (1973) *Chem. Phys. Lipids 10*, 127–136.
Verger, R., & deHaas, G. H. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77–117.
Waite, M. (1987) *Handb. Lipid Res. 5*, 92–98.
Waite, M., Thuren, T., Wilcox, R. W., Sisson, P., & Kucera, G. L. (1991) *Methods Enzymol. 197*, 331–339.
Weisgraber, K. H. (1990) *J. Lipid Res. 31*, 1503–1511.
Weisgraber, K. H., & Mahley, R. W. (1980) *J. Lipid Res. 21*, 316–325.
Weisgraber, K. H., & Shinto, L. (1991) *J. Biol. Chem. 266*, 8651–8654.
Weisgraber, K. H., Rall, S. C., Jr., & Mahley, R. W. (1981) *J. Biol. Chem. 256*, 9077–9083.
Weisgraber, K. H., Rall, S. C., Jr., Mahley, R. W., Milne, R. W., Marcel, Y. L., & Sparrow, J. T. (1986) *J. Biol. Chem. 261*, 2068–2076.
Wetterau, J. R., Aggerbeck, L. P., Rall, S. C., Jr., & Weisgraber, K. H. (1988) *J. Biol. Chem. 263*, 6240–6248.
Zannis, V. I., & Breslow, J. L. (1981) *Biochemistry 20*, 1033–1041.