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THE EFFECT OF PHOSPHOLIPIDS ON APOB CONFORMATION AND STABILITY IN RECONSTITUTED LOW DENSITY LIPOPROTEINS

by

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Abstract

This thesis is concerned with the mechanism that underlies the apparent atherogenic capacity of low density lipoproteins (LDL). Utilizing a novel reconstitution approach, this work provides a means for determining the exact effects of specific LDL lipids on the conformation of the protein moiety of LDL, apolipoprotein B (apoB). Specifically, it examines the relationship between LDL phospholipids and apoB conformation and stability. Through an independent understanding of the lipids associated with apoB we will be able to gain insight into the structural organization of the protein in intact LDL particles and as well the functional aspects of the lipoprotein.

Interactions of apolipoproteins with phospholipids not only define their structures but may also determine the metabolic fates of the lipoprotein complexes. In order to elucidate the effect of phospholipids on apoB conformation and stability, reconstituted LDL (rLpB) particles were prepared from apoB and palmitoyloleoyl phosphatidylcholine (POPC). Intact apoB was isolated from native LDL using sodium deoxycholate to solubilize the LDL lipids and size exclusion chromatography to separate apoB from lipids and detergent. Compositional analysis revealed that apoB was completely devoid of detergent, phospholipid, cholesterol and cholesteryl esters but had retained ~70% of the triglyceride (TG) from LDL. Aqueous soluble apoB-TG complexes exhibited a significantly reduced amphipathic α-helical content (17%) and net negative charge (-2.9 mV) as compared to LDL-bound apoB (49% and -6 mV, respectively). Reconstitution of apoB-TG with phospholipid was accomplished through spontaneous complexation with POPC vesicles. In contrast to the inability of exchangeable apoproteins such as apoA-I to spontaneously form lipoprotein particles from POPC vesicles, apoB-TG was able to rapidly (<10 min.) solubilize POPC at temperatures ranging from 4 to 24°C. Electrophoresis on 4-15% gradient acrylamide gels showed the rLpB particles to be homogeneous and to exhibit a single discrete band. Circular dichroic spectroscopy identified major

changes in the secondary structure and stability of apoB with the addition of POPC. Inclusion of 300 molecules of POPC significantly increased the α-helical content of apoB to 34% and the net negative charge to -4.9 mV. Phospholipidation of apoB also significantly decreased the effectiveness of GdnHCl in unfolding the apoprotein. While the α-helices in apoB-TG could be completely unfolded to random coil in the presence of 5.5 M GndHCl, POPC/apoB complexes were only slightly denatured and exhibited a 5% reduction in α-helicity at the same GdnHCl concentration. Similar observations were seen with native LDL, where apoB was only partially denatured in 5.8 M GdnHCl. The immunoreactivity of rLpB was assessed through a competitive radioimmunoassay using various monoclonal antibodies. Unexpectedly, it was found that apoB-TG was immunoreactive with antibodies 1D1, 2D8, 3F5, 4G3 and 5E11. This suggests that interactions between apoB and triglyceride may play an important role in the stabilization of apoB tertiary structure. The inclusion of 300 molecules of POPC significantly increased the immunoreactivity of the conformation specific antibodies 2D8 ($p<0.001$) and 4G3 ($p<0.01$) but had no major effect on the epitope accessibility or affinity of the other monoclonal antibodies studied. Overall, these data suggest that triglyceride-bound apoB will spontaneously solubilize POPC vesicles and undergo alterations in conformation, stability and charge similar to that observed for apoB on LDL. Structural changes are specific to distinct α-helical domains in apoB. Reconstitution of apoB-TG with POPC does not completely regenerate the ability of apoB to interact with all monoclonal antibodies with the same immunoreactivity as that of LDL.

Dedications

I dedicate this work to my loving parents

and brother, Vijay.

Acknowledgments

I would like to express my appreciation to everyone who has contributed to the successful completion of my education over the last few years of my graduate studies. Particularly, I would like to thank my supervisors, Dr Ross Milne and Dr Dan Sparks who have provided me with the opportunity to explore and develop my research skills. Each in there own way has taught me the meaning of critical thinking and brought new insights into my research project.

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ABREVIATIONS

apoB100	ApolipoproteinB100
VLDL	Very low density lipoproteins
LDL	Low density lipoproteins
IDL	Intermediate density lipoprotein
HDL	High density lipoprotein
TG	Triglyceride
PL	Phospholipid
CH	Cholesterol
CHE	Cholesterol ester
DMPC	Dimyristoyl phosphatidylcholine
POPC	1-palmitoyl-2-oleoyl phosphatidylcholine
GGE	Gradient gel electrophoresis
GdnHCl	Guanidine Hydrochloric acidl
SDS	Sodium dodecyl sulfate
mAb	Monoclonal antibody
apoB-TG	Apolipoprotein B associated with triglyceride
nLDL	Native low density lipoprotein
rLpB	Reconstituted low density lipoprotein

Introduction

1.1 Low density Lipoproteins (LDL) in Atherogenesis:

Atherosclerosis is a multifactorial disease involving many etiological factors with harsh ramifications and intricacies that have yet to be understood. Four factors appear to contribute to the development of high serum cholesterol levels: (1) aging, (2) diet, (3) genetic predisposition, and (4) secondary causes (Havel R.J., Goldstein J.L. and Brown M.S.,1980). While direct evidence is lacking which correlates atherosclerotic risk factors and the bio-molecular events involved, much information has been reported which links altered plasma lipid metabolism to the development of atherosclerotic plaques (Chobanian A.V., 1992).

Atherosclerosis results from plaque growth and inevitably results from the failure of the vessel wall to dilate in response to reduced blood flow. Clinical and experimental studies have established that elevated plasma concentrations of low density lipoproteins that have undergone oxidative modification are strongly associated with the development of atherosclerosis and its sequel, coronary artery disease (Ross R., 1993). The endothelium appears to play an important role in the events that occur early in atherogenesis. It has been shown that hypercholesterolemia, can cause changes in endothelial cell morphology and function. The endothelial cells represent a unique monolayer of cells that have strict growth requirements; they attach to one another and to the underlying matrix by a series of cell surface integrin molecules that bind to collagen or other matrix molecules. Endothelial cells, when injured, are unable to regenerate the wound site. Consequently over time, there may be loss of endothelial cells at local sites, concomitant with the loss of normal physiologic properties provided by the endothelium. If this occurs, these sites might become thrombogenic or more adhesive for leukocytes, which would result in an increase in lesion formation. Data demonstrates that changes in the endothelium associated with atherogenesis results in the attachment and subsequent

adherence of leukocytes mediated by a series of cell-surface, adhesive glycoproteins (Schwartz S.M., Murry C.E., O'Brian E.R., 1996). One of the earliest changes in the endothelium is seen in its permeability, wherein increased amounts of lipoprotein are transported by the endothelium and localize in the subendothelial space of the intima. During transcytosis, many of these lipoprotein particles may be modified by oxidation or glycation by the endothelium. The formation of oxidized LDL may induce expression of genes that cause the endothelial cells to form chemotactic molecules, additional cell adhesion, and possibly growth-regulatory molecules or cytokines. These changes may ultimately lead to monocyte endothelial attachment, adherence, and transmigration so that the monocytes enter the subendothelium, become activated as macrophages, and are joined by T lymphocytes. Macrophages are a rich source of growth-regulatory molecules and cyokines, and as such may be the principal mediator of cell migration and proliferation within the lesions of atherosclerosis. Macrophages are also the principal source of foam cells in the lesions because they take up lipid and oxidized LDL through scavenger receptors or bulk phase endocytosis and a putative oxidized LDL receptor. Macrophages can oxidize LDL through various activities, including the formation of lipoxygenase and nitric oxide. The fatty acids undergo peroxidation, become covalently cross-linked to the apolipoprotein B moiety of the LDL particles, and are taken up via the scavenger receptors to localize in the macrophage. The uptake of oxidized LDL and other substances within the lesions of atheroclerosis may serve as a stimulus to induce gene expression for several growth regulatory molecules and cytokines, which can be chemotactic agents, growth agonists, or antagonists. The macrophage can make agents that induce monocyte proliferation, smooth muscle and endothelial proliferation. Activated macrophages can also produce a series of chemotactic molecules for other monocytes, for endothelieal cells and for smooth muscle cells. Although macrophages do not exhibit appreciable binding of native LDL, they possess scavenger receptors that

bind to oxidized LDL. Unregulated uptake of oxidized LDL through scavenger receptors present on macrophages results in the formation of foam cells. The foam cell is the hallmark of the atherosclerotic lesion (Ross R., 1993). Foam cells eventually accumulate to form fatty streaks in the intima of an artery. Fatty streaks do not produce significant obstruction of the arterial lumen but are eventually transformed into the next stage of the atherosclerotic lesion, the fibrous plaque. The fibrous plaque is a proliferative lesion in which a fibrous cap covers a lipid core. Smooth muscle cells are present that produce fibrous connective tissue containing ground substance, collagen fibers, and reticulum fibrils. As the lesions accumulate increasing numbers of cells, and the macrophages scavenge the lipid, some of the macrophages may emigrate back into the blood stream by pushing apart the endothelial cells. Sites at such branches and bifurcations may become thrombogenic, and platelet mural thrombi may form. Platelets within the thrombi can release many potent growth-regulatory molecules into the artery which are added to those released by the activated macrophages and possibly by lesion smooth muscle cells. Platelet thrombi can also form at sites where endothelial dysjunction has occured. Ultimately, the formation and release of numerous growth-regulatory molecules and cytokines develop into a network of activated macrophages, smooth muscle, T cells and endothelium and lead to progression of the atherosclerotic lesion to a complicated lesion. These changes set the stage for the final complications of hemorrage into the plaque, causing plaque rupture, thrombosis and vascular occlusion. However, the exact cellular and molecular mechanisms of the latter stages of atherogenesis remain unclear.

1.2 Lipoproteins:

1.2.1 Lipoprotein Structure and Class:

The major lipid species of human plasma are the phospholipids, cholesterol, triglycerides, and cholesteryl esters (Morrisett J.D., et al., 1977). These insoluble species are transported in blood as macromolecular complexes of lipids and proteins known as plasma lipoproteins. The plasma lipoproteins are well recognized transport forms of lipid in the circulation. They may be viewed as micellar structures which are in a state of dynamic equilibrium with respect to each other as well as with various tissues and membrane compartments within the body (Brown M.S., Kovanen P.T. and Goldstein J.L., 1981). A general structure for normal plasma lipoprotein particles has been proposed in which the neutral lipids, cholesteryl esters and/or triglycerides, are separated from the external aqueous environment by a surface monolayer, consisting of the apolipoproteins and the polar lipids which are mostly phospholipids.

The plasma lipoproteins consist of five major classes and several subclasses (Hamilton R.L., Moorehouse A., and Havel R.J., 1991) Each class, as separated by ultracentrifugation, is heterogeneous in size, density and composition (Chen C G., et al., 1994.and Krauss M.R. and Burke J.D., 1982). The largest of the particles are chylomicrons. The chylomicrons are transient particles, which are synthesized in the gut and carry dietary triglycerides and cholesterol. They contain triglyceride as their major lipid constituent, although their content of cholesteryl ester may be very important in regulating the hepatic synthesis of cholesterol. The very low density lipoprotein (VLDL), with their major lipid component of endogenous triglyceride and principal apolipoprotein of B100 are synthesized in, and secreted by, the liver after delivery of unesterified fatty acids. The third class, intermediate density lipoproteins (IDL), represent an intermediate in the conversion of VLDL to LDL by lipoprotein lipase. The IDL contain relatively less triglyceride and cholesteryl ester

compared to VLDL. LDL are the major carriers of cholesterol and cholesteryl ester in the plasma. Approximately 60% of the cholesterol is transported as LDL in man and about three-fourths of this cholesterol is in the esterified form. The last class of lipoproteins are the HDL particles. The lipids of HDL are primarily phosphatidylcholine and cholesteryl esters. HDL are much richer in protein and contain approximately one-half protein and one-half lipid by weight and are usually subdivided into at least two size subclasses, HDL_2 and HDL_3.

*1.2.2 **Lipoprotein Metabolism:***

The liver plays a major role in the maintenance of cholesterol homeostasis across individual organs and for regulating the steady state concentration of low density lipoprotein cholesterol in the plasma (Young S.G., 1990). Dietary cholesterol is delivered to the liver in the form of chylomicron remnant particles. The exogenous pathway begins with dietary consumption of fat and cholesterol. After dietary fat is partially hydrolyzed in the intestine, particles called chylomicrons, which incorporate dietary triglycerides are synthesized. Chylomicrons are produced by the intestinal mucosal cells from dietary lipids and secreted into the mesenteric lymph ducts, from which they enter the plasma. Apolipoproteins C-II, C-III, and E and cholesteryl ester are transferred from HDL to chylomicrons in both lymph and plasma. Apo C-II activates endothelial lipoprotein lipase, which hydrolyzes the chylomicron core triglycerides. The resulting free fatty acids are taken up by adipose tissue for storage and by muscle for energy. The chylomicron shrinks in size and is known as a remnant particle. Normally chylomicron remnants are present in the plasma for only a matter of minutes (Ginsberg H.N., 1990). The residual chylomicron remnants are relatively enriched with cholesteryl ester, both from the loss of triglyceride and from addition of cholesteryl ester from HDL. Apolipoproteins B-48 and E remain on the surface of chylomicron remants. These remnants are rapidly removed from the plasma by the liver, where a receptor binds to apoE. The exact nature of

the receptor is unknown, but it does not appear to be the same receptor that binds to low density lipoproteins. Within hepatocytes, the particle is degraded to its constituents through the LDL pathway (Brown M.S., et al., 1981). The liver either disposes of the cholesterol by converting it to bile salts that are excreted into the intestines, or diverts it back into the plasma in the form of newly secreted lipoproteins.

LDL is thought to be primarily responsible for the delivery of cholesterol to extrahepatic tissues (Brown M.S., et al., 1981). The liver is capable of producing LDL indirectly via VLDL. VLDL is a large triglyceride-rich lipoprotein. VLDL triglyceride is made from glycerol and fatty acids that have been either released by adipose tissue or synthesized in the liver; VLDL cholesterol comes from circulating lipoproteins or hepatic synthesis. VLDL particles contain phospholipids and apolipoproteins B-100 (one molecule per particle), CI, CII, CIII, and E, but VLDL acquires additional C apolipoproteins and apo E in plasma. Once secreted from hepatocytes, VLDL interacts with lipoprotein lipase in capillary endothelium, apo CII acts as a cofactor for this reaction. The interaction with lipoprotein lipase causes the hydrolysis of core triglycerides to produce fatty acids for adipose and muscle tissue. VLDL remnants can be taken up by the liver or be further hydrolyzed to produce intermediate density lipoproteins (IDL). IDL retains apoproteins B100 and E, and is enriched in cholesteryl ester relative to triglyceride. IDL is gradually converted by hepatic triglyceride lipase to smaller, denser, cholesteryl ester-rich LDL. As IDL is being converted it loses apolipoprotein E and only retains apolipoprotein B. LDL normally carries about 75 percent of the circulating chlolesterol, transporting it to extrahepatic cells for steroid hormone and cell membrane synthesis. It normally circulates for two to three days and about half is taken up by the liver. Approximately 70% of LDL is cleared by receptor uptake, and the remainder is removed by scavenger cell pathways using non receptor mechanisms.

1.3 Low Density lipoproteins (LDL):

1.3.1 Structure and Heterogeneity:

Low density lipoproteins are a heterogeneous group of particles of 25-29 nm in diameter with a molecular weight of 2 million Da. (Schumaker V.N, Phillips M.L. and Chatterson J.E, 1994). LDL are the major cholesterol-carrying lipoprotein of human plasma. The average LDL size and density are found to vary between individuals and studies suggest that the variation is due to both genetic and dietary factors. The core of LDL particles consist mostly of cholesterol esters (48% of the total lipid fraction) and triacylglycerols (6%). The core is surrounded by a layer of phospholipid (34%), unesterified cholesterol (12%), together with a single apoprotein, apoB100 (Figure 1). It is believed that the protein moiety of LDL is present at the surface of LDL particles to stabilize the structure of the apolipoprotein-lipid particular complex. Structurally, LDLs are well described as emulsion particles (Schumaker V.N., et al., 1994). An emulsion may be defined as one liquid embedded in another and kept in solution by emulsifing agent. For LDL, the first liquid is a droplet of oil, largely cholesteryl ester but containing some triglyceride; the second liquid is the aqueous plasma and the emulsifing agent is a monolayer of phospholipid, unesterified cholesterol, and protein. This monolayer forms an amphipathic surface coat surrounding the oil droplet and separating the hydrophobic, liquid core of the LDL from the aqueous plasma. The emulsion particle model also places apoB at the surface of the LDL, consistent with proteolysis studies, and with studies of the binding of anti-apoB monoclonal antibodies to LDLs (Chan L., 1992).

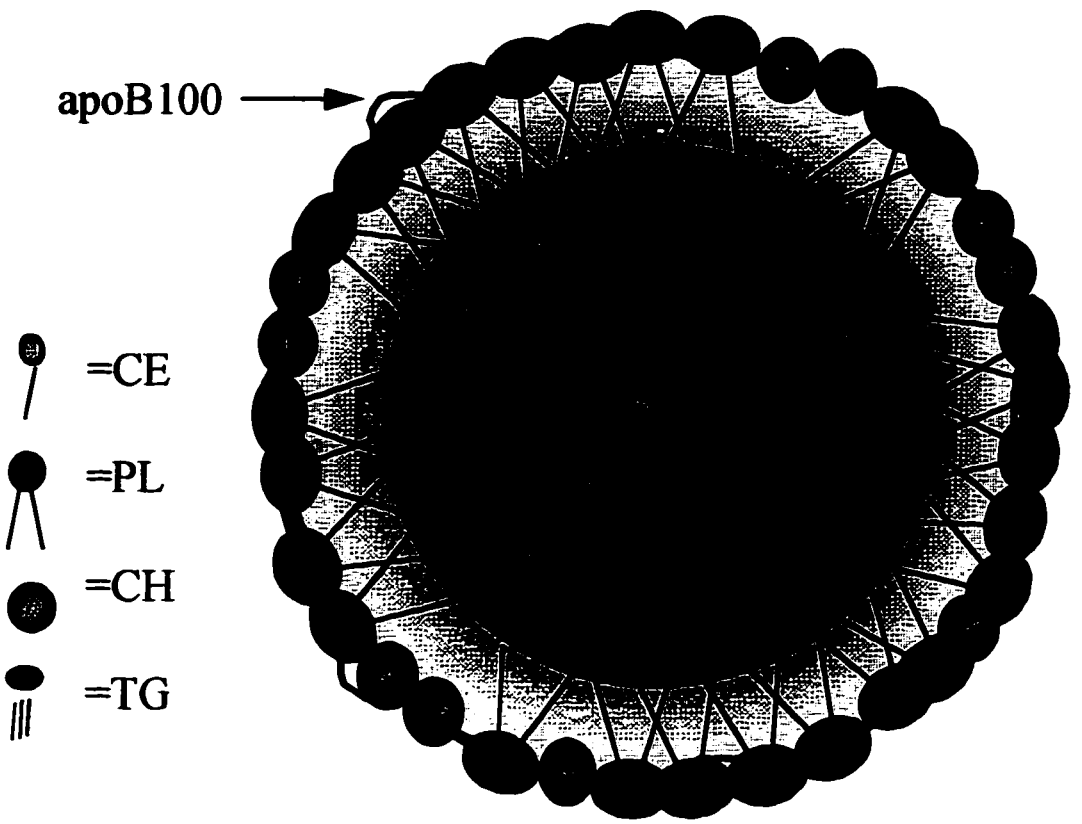

Figure 1: Structure of low density lipoprotein with component cholesteryl esters (CE), phospholipids(PL), cholesterol (CH) and triglycerides (TG) and apoB100.

Low density lipoproteins are a continuum of particles distributed over the density range from 1.006-1.063 g/ml (Chen Chi G., Liu Weiquin, Duchateau Philippe, Allaart Jeanne, Hamilton L. Robert, Mendel M. Carl, Lau Kenneth, Hardman A. David, Frost H. Philip, Malloy J. Mary and Kane P. John. 1994). Seven LDL size species have been identified within four density subgroups on the basis of distribution of LDL size as determined by nondenaturing gradient gel electrophoresis. These particles differ markedly in their physical, chemical, hydrodynamic, and immunological properties. The bulk of LDL is present within the 1.019 to 1.063 g/ml density interval, while the less dense region of this distribution (i.e., d 1.006-1.019 g/ml) is now equated with the IDL subfraction. In most

individuals the LDL subclass pattern can be categorized as pattern A or pattern B (Austin A.M., Breslow J.L., Hennekens C.H., Buring J.E., Willett W.C., Krauss R.M, 1988). Pattern A is characterized by the predominance of larger, more buoyant LDL particles. Pattern B is characterized by a preponderance of small dense LDL particles, and is associated with a threefold increased risk of myocardial infarction, independent of age, sex and relative weight (Austin A.M., et al., 1988).

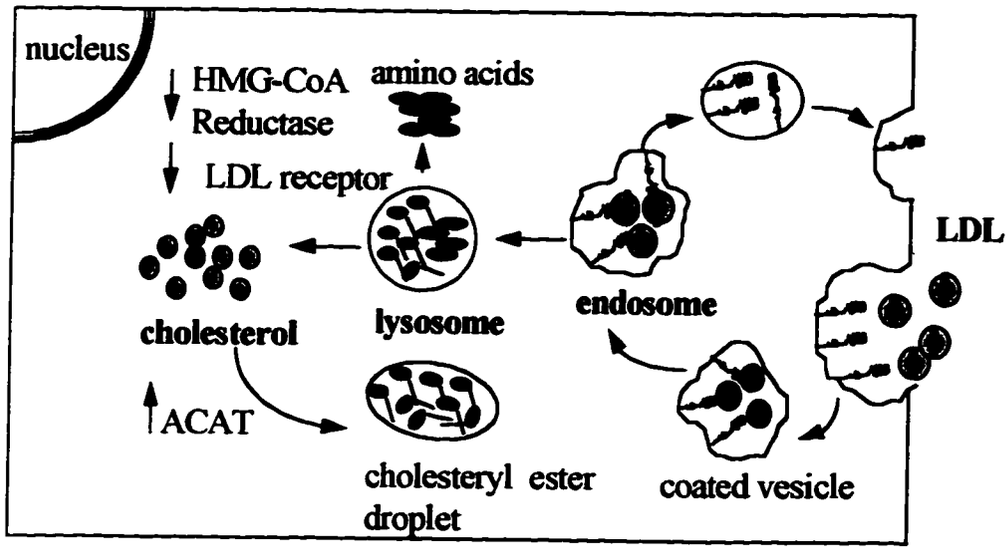

Figure 2: Receptor mediated uptake of LDL: pathways for uptake and degradation of LDL at the cellular level.

1.3.2 Fates of LDL:

The removal of LDL from the plasma is mediated by the high affinity LDL pathway (Figure 2) (Brown M.S., et al., 1981). The rate of uptake of LDL by this pathway is a function of the number of LDL receptors; the lipoprotein first binds to the LDL receptor; it is then internalized and digested by lysosomes. In the lysosomes, cholesterol esters are hydrolyzed, and cholesterol is released into the cytoplasm for use in cell membranes. When amounts of unesterified cholesterol exceed the need for

cell membranes, the excess cholesterol inhibits the cells' own synthesis of cholesterol, and it can be stored in the cell as inert cholesterol ester. If the cell accumulates an excess of cholesterol, synthesis of LDL receptors also is reduced, thereby reducing uptake of LDL-cholesterol (Brown M.S., et al., 1981).

Second, LDL can be removed from the circulation via a nonreceptor pathway (Grundy S.M., 1990). Several types of nonreceptor uptake are possible, these include bulk-phase phagocytosis, endocytosis by the reticulendothelial system, and possibly other types. Current estimates indicate that approximately 75% of serum LDL is cleared by the liver, with the remaining 25% by extrahepatic tissues. Uptake of LDL by either the liver or extrahepatic tissues can occur by both receptor and non receptor pathways. In general, the latter are poorly defined, nonspecific pathways that clear plasma proteins. Apparently, two thirds to three fourths of the circulating pool of LDL is cleared by receptor pathways, whereas one-fourth to one third is removed via nonreceptor pathways. On average, 30% to 40% of the total plasma pool of LDL is removed each day.

1.3.3 General Regulation of Serum-LDL Levels:

The immediate precursor of LDL is the VLDL remnant, which in turn is derived from VLDL. The amount of LDL produced depends on two factors: (1) the quantity of VLDL produced by the liver and (2) the fraction of VLDL remnants removed directly by the liver (Grundy S.M., 1990). The latter is determined in part by the number of LDL receptors, since VLDL remnants can be removed by LDL receptors. The concentration of LDL also is determined by the rate of its removal from the circulation, either by the liver or by extrahepatic tissues. Again, LDL receptors are largely responsible for clearance of serum LDL. Thus, the number of LDL receptors is a key regulator of serum LDL concentration by affecting both the rate of formation and the rate of clearance of LDL (Grundy S.M., 1990).

1.3.4 ApoB Structure and Function:

Apolipoprotein B is one of the largest and most abundant of the human plasma apolipoproteins. It exists naturally in two forms, apoB100 and apoB48. It is composed of 4536 amino acids and is crucial for the assembly of triglyceride-rich VLDL particles. ApoB100 is synthesized in the liver and is virtually the only protein of LDL. The apoB100 of LDL serves as a ligand for LDL receptor mediated uptake of LDL particles by the liver and extrahepatic tissues. The LDL receptor binding region of apoB100 is located in the carboxy-terminal portion of the molecule, whereas its lipid-binding regions appear to be broadly dispersed throughout its length (Chan L., 1992).

ApoB48 contains the amino terminal 2152 amino acids of apoB100 and is produced by the intestine as a result of editing of a single nucleotide of the apoB mRNA, which changes the codon specifying apoB100 amino acid 2153 to a premature stop codon (Chan L., 1992). ApoB48 has an obligatory structural role in the formation of chylomicrons; therefore, its synthesis is essential for delivery of dietary fats and fat-soluble vitamins.

An elevated level of apoB100 in the plasma is a potent risk factor for developing premature atherosclerortic disease (Chan L., 1992). Many different apoB gene mutations that affect the concentrations of both apoB and cholesterol in the plasma have been characterized. A missense mutation in the codon for apoB100 amino acid 3500 is associated with hypercholesterolemia (Chan L., 1992). This mutation results in poor binding of apoB100 to the LDL receptor, thereby causing the cholesteryl ester-enriched LDL particles to accumulate in the plasma. This disorder is called familial defective apoB100, and it is probably a cause of premature atherosclerotic disease. Familial hypobetalipoproteinemia is a condition associated with abnormally low levels of apoB and cholesterol; affected individuals may actually have a reduced risk of atherosclerotic disease (Chan L.,

1992). Hypobetalipoproteinemia can be caused by a number of different apoB gene mutations that interfere with the translation of a full-length apoB100; the syndrome can also result from apoB alleles that yield reduced amounts of full length apoB100. Existing evidence suggests that neither familial defective apoB100 nor familial hypobetalipoproteinemia is particularly rare (Chan L., 1992).

The gene for human apoB100 is located on chromosome 2. Region mapping and in situ hybridization have positioned the gene to the short arm in the p23 and p24 region (Chan L., 1992). Thus, the apoB gene is unlinked to members of the gene family encoding the other major apolipoprotein species, which are dispersed on chromosomes 1, 11 and 19. Twenty-eight introns interrupt 29 exons and the gene spans 43 kbp of genomic DNA. The first exon contains the 5'-untranslated region and so codes for the signal sequence. The mature protein sequence starts with exon 2. Most of the exons range in size from 150 to 250 nucleotides, and 24 of these encode the first one-third of apoB. Exon 26 is composed of 7572 base pairs and is by far the longest reported for a vertebrate genes. The 1st exon, exon 29, is also very long, and contains 1906 bp. Complete sequences of all 29 exons and all but the middle regions of about a dozen large introns have been reported. The intron/exon boundaries have all been sequenced and conform to the standard pattern for these junctions (Chan L., 1992).

The major structural features of apoB100 are as follows: there are 19 potential N-linked glycosylation, sites for which only 16 are accurately glycosylated. These sites are unevenly distrubuted; 8 of the 16 occur within 700 residues (between Asn 2752 and Asn 3438) in the C-terminal half of the molecule. ApoB100 contains 8-10% carbohydrate in the form of galactose, mannose, N-aceytlyglucosames and sialic acid residues there are 25 cysteine residues in apoB100 that

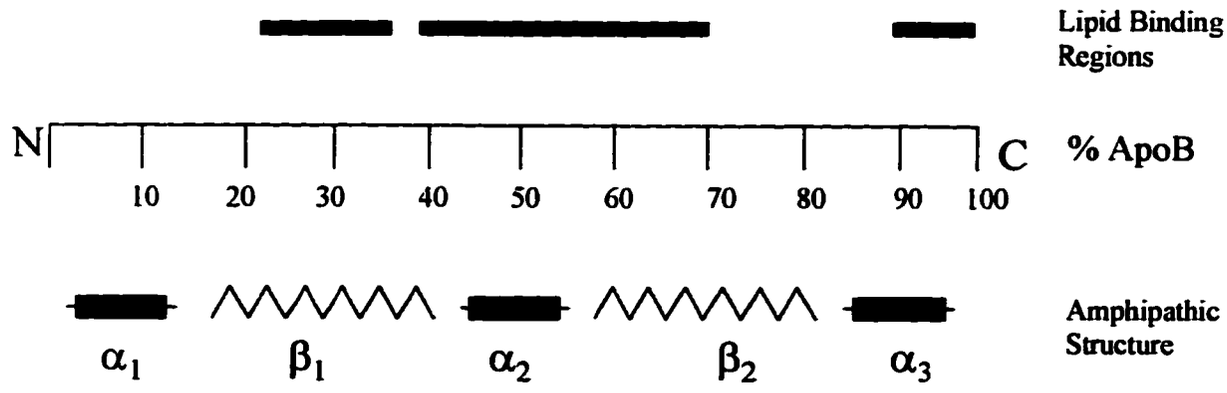

Figure 3: **Postulated lipid-binding regions and predicted secondary structures of apoB100**

are also quite asymmetric in distribution with a concentration in the N-terminal region. Sixteen of the cysteine residues are involved in disulfide linkage (Chan L., 1992)

Essentially all circulating apoB100 is associated with lipid. Information about the secondary structure of apoB can help elucidate the assembly of LDL particles. Steele and Reynolds (Steele J.C.H., and Reynolds J.A., 1979) used the method of Greenfield and Fasman (Greenfield N. and Fasmann G.D., 1969) to analyse the secondary structure of apoB in LDL and found the structure to include 25% α-helix and 40% β-structure; the remaining 35% of the structure was random. Other reports have estimated a higher α-helix (44%) and lower β-sheet (16%) content (Goormaghtigh E., De Meutter J., Vanloo B., Brasseur R., Rosseneu M., and Ruysschart J.M., 1989). Use of an algorithm for secondary structure predicts that apoB contains 43% α-helix , 21% β-sheet structure, 20% random coil structure, and 16% β-turns confirming the previous report (Chan L., 1992).

Recently Segrest et al. (Segrest J.P., Jones M.K., Mishra K.V., Anatharamiaah G.M., Garber D.W., 1994) proposed a pentapartite model for human apoB structure in which apoB has a five domain folding organization composed of three amphipathic α-helical domains alternating with two

amphipathic β-strand domains (NH_2-α_1-β_1-α_2-β_2-α_3-COOH) (Figure 3). Amphiphathic helix cluster I contains many disulfide bonds and represents one or more folded α-helical globular domains that can, under certain circumstances, associate with lipid. The two lipid-associating amphipathic helical clusters II and III, may represent reversible, and thus flexible, lipid-associating domains, perhaps accounting for the presence of several subclasses of LDL of different sizes. Finally, the gap region that contains extensive amphipathic β-strand represents irreversible lipid associating domains.

1.3.5 Assembly of VLDL:

It is generally believed that the assembly of VLDL occurs cotranslationally within the lumen of the endoplasmic reticulum where lipids are recruited by apoB in the presence of a microsomal trigyceride transfer protein (MTP) (Innerarity L.T., Boren J., Yamanaka S. and Olofsson S.O., 1996). MTP acts as a mediator that facilitates the association of lipids with protein (Innerarity L. et al., 1996). It functions to transfer triglycerides between amphipathic surfaces of liposomes. ApoB100 and apoB48 are synthesized on attached ribosomes of the endoplasmic reticulum. When nascent chains reach a length of 700-900 amino acids, lipoprotein assembly begins. The N-terminal region of apoB contains twelve cysteine residues that are capable of forming disulfide bonds. Formation of disulfide bonds results in a folded protein that is water soluble and globular and incapable of interacting with lipids. The N-terminal domain of apoB also has sites that have an affinity for proteins that are capable of lipidating apoB such as MTP. The amount of lipid added to apoB is directly correlated to apoB length and proteins involved in lipid assembly. The absence of these proteins may result in the degradation of apoB due to an increased sensitivity to proteases (Innerarity L. et al., 1996).

Newly synthesized phospholipids (mainly phosphatidylcholine) seem to be added to the nascent particle during the translation/translocation process. Phospholipids are a necessity for the

secretion of the assembled LDL. The addition of triglycerides is an essential component for the formation of lipoprotein particles. The forming triglyceride droplet arises from a storage pool in the cytoplasm. It is first hydrolyzed to diacylgycerol and then reesterified to triglyceride before assembly. MTP acts to transfer lipids, mainly triglycerides but also phospholipids and cholesterol esters between amphipathic surfaces of liposomes (Innerarity L. et al., 1996).

The first step of apoB48 assembly is independent from the second step (addition of lipid core). ApoB48 undergoes, in a first step of apoB48 VLDL assembly, an MTP-dependent co-translational lipidation to form a lipoprotein with HDL size and density. In the second step, these particles are converted to large triglyceride-rich apoB48 containing VLDL/chylomicron particles (Innerarity L. et al., 1996).

1.4 Recombinant lipoprotein systems:

The basis for our present knowledge of protein structure has developed rapidly and has stemmed from electron microscopy, X-ray and neutron structural studies and nuclear magnetic resonance. However, due to the molecular complexity of lipoproteins, it has been difficult to correlate the various constituents of a lipoprotein particle to its function using the above methodology. Thus the ability to disassemble and reassemble LDL complexes provides a useful means for studying the molecular architecture and interactions in lipoproteins. Additionally, these model systems provide a mean to establish the consequences of changes in lipoprotein composition and structure on the metabolic processes in which lipoproteins are involved.

The major apolipoprotein of LDL, apoB, has proved difficult to isolate and characterize due to its high hydrophobicity, strong tendency to form aggregates and insolubility in aqueous media (Kreiger M., 1986). Nonetheless, a variety of solubilization methods employing detergents, chemical modifications, denaturants have been developed in order to maintain apoB in solution. Detergents

which can effectively dissociate lipids from apoB include sodium dodecyl sulfate, triton X-100 and sodium deoxycholate (Kreiger M., 1986). Each of these detergents provides an effective means for weakening the interactions of lipids from protein. The detergents act to prolong the stability of the apoB in the absence of lipids. The choice and concentration of detergent to be used is usually dependent on the degree of apoB dissociation from lipids. Sodium deoxycholate is a weakly ionic detergent and has been extensively used by investigators for the solubilization of apoB, since it dissociates interactions between protein molecules effectively (Watt M.R. and Reynolds A. J., 1981). Organic solvents have also been employed by several investigators in the delipidation of LDL (Kreiger M., 1986).

Reconstitution of LDL-like complexes is a difficult and delicate procedure and has been accomplished to different degrees using a variety of methodologies. In the late seventies, studies by Krieger et al (Kreiger M., 1986) showed that reconstituted spherical LDL particles could be produced by extracting and replacing the neutral lipid core of LDL after stabilization of the particle apoB on potato starch powder. Investigations have shown that reconstituted LDL prepared by this method has very similar physical characteristics (composition, surface charge and apoB epitope expression) to authentic LDL and also resemble LDL in their ability to interact with the LDL receptor (Kreiger M., et al.). In the early eighties, Ginsburg and colleagues (Ginsburg S.G., Small D. and Atkinson D., 1982), followed by Lundberg et al., (Lundberg O., and Suominen L., 1984), further showed that LDL could also be reassembled from sodium deoxycholate-solubilized apoB and sonicated microemulsions of egg yolk phosphatidylcholine (EYPC) and cholesterol oleate (Ginsburg S.G. et al., 1982). This methodology was more versatile than the potato starch technique as it permitted the investigators to vary all of the surface and core lipid components, while the starch technique only allowed for changes in neutral lipid components of the recombinant particles. Characterization of the reassembled LDL

complexes showed these particles to also have similar physical properties to authentic LDL (Ginsburg G.S. et al., 1982).

Overall, these methods of reconstitution have proven successful in providing a way for preparing biologically active particles with compositions similar to native LDL. However, these investigators have not examined in depth the effects of specific lipids in isolation on LDL structure and integrity in order to understand the mechanism underling the association of apoB with lipid.

1.5 Rationale for this study:

LDL heterogeneity has been a consistent hindrance to the understanding of the architectural integrity of these lipoproteins. In part it arises from the transformation of VLDL to LDL in plasma through lipolysis and results in the creation of various LDL particles which differ in lipid composition, charge, size and in apoB conformation which in turn, is manifested in changes in the immunoreactivity, protease sensitivity and receptor-binding properties of the particles. Therefore, even though it is known that alterations in LDL lipid composition give rise to specific changes in the conformation and charge on apoB which closely correlate to an altered LDL function, how these specific factors act in isolation to alter LDL integrity have yet to be identified. Various investigators have been unsuccessful in correlating specific LDL variables involved in altering apoB conformation due to limitations in methodologies and their inability to control for LDL heterogeneity. As a consequence there is controversy in the current literature concerning the ability of core composition of LDL to modulate LDL function.

Several classes of LDL have been identified and classified into two levels, pattern A and pattern B. Pattern A is classified by the predominance of larger, more buoyant LDL particles. Pattern B is characterized by predominance of small LDL particles and is associated with increased triglyceride and reduced HDL cholesterol levels and an increased risk of coronary artery disease

(Austin M.A. et al., 1988). A correlation has been found between differences in receptor-mediated LDL catabolism and subjects with pattern A and pattern B LDL. However, the exact nature of this correlation remains questionable due to inconsistency in findings. Swinkels et al., (Swinkels D.W., Hendriks J.C.M., Demacker P.N.M., and Stalenhoef A.F.H., 1990) found no differences in the receptor binding of LDL subfractions to LDL receptors on fibroblast and HepG2 cells, but other studies found that both buoyant and dense LDL subfractions had reduced binding compared with medium density LDL subfractions (Swinkels D.W., Demacker P.N.M., and Hak-Lemmers H.L.M., 1988 and Chappell D.A., Fry G.L., Waknitz M.A., Berns J.J., 1991). Still other studies (Campos H., Arnold K.S., Balestra M.E., Innerairity T.L, and Krauss R.M., 1996) have found greater binding affinity for buoyant LDL subfractions compared with medium and dense LDL subfractions. Therefore, further investigations must be performed to resolve this controversy using a methodology which minimizes LDL heterogeneity.

Kinoshita et al., (Kinoshita, M., Krul, E.S., and Schonfeld S., 1990) have shown that modification of core lipids of LDL produces selective alterations in the expression of apoB epitopes. In this study, the investigators altered the core composition of LDL *in vitro* by incubating LDL with VLDL in the presence of partially purified human plasma lipid transfer proteins. The conformation of apoB was then monitored by probing epitope expression and by examining interactions between LDL and LDL receptors on human fibroblasts. During the incubations with VLDL and lipid transfer proteins, the diameters of LDL decreased with a corresponding increase in triglycerides. Increase in the triglyceride was manifested by a progressive decrease in immunoreactivity with antibodies distant from the putative receptor binding site. Receptor binding affinity decreased with increasing LDL triglycerides even though epitopes near the receptor binding region were not affected. These data suggest a possible relationship between LDL core composition and apoB conformation.

In another study by Aviram et al., (Aviram M., Bierman E.L., and Chait A., 1988) it was found that modification of LDL by lipoprotein lipase or hepatic lipase induced an enhanced uptake and cholesterol accumulation in macrophages. Both lipases modified LDL by substantially reducing core triglyceride content with no marked changes in size, charge or lipid peroxide content in comparison to native LDL It was found that triglyceride content of the core of LDL affected the chemical and physical properties of the lipoprotein surface thereby modifying the interaction of apoB with the LDL receptor.

McKeone et al., (McKeone, B.J., Patsch, J.R., and Pownall, H.J., 1993) also showed that plasma triglycerides determine LDL physical properties and cell specific binding in cultured cells. This investigation compared triglyceride-rich LDL, produced by *in vitro* lipid transfer or isolated from hypertriglycerdemic subjects to normal LDL. Overall, it was found that there were no differences in circular dichroic spectra but normal and triglyceride rich LDL differed in reactivity with a mAb specific for an epitope near the amino terminus of apoB, in accessibility of protease cleavage sites and in surface charge. Triglyceride enrichment was found to also decrease the uptake of LDL in fibroblasts but increased in HepG2 cells.

In contrast to the above studies in which it was concluded that LDL core composition is a primary determinant of apoB conformation and function, other studies have found that the ability of LDL to react with the LDL receptor of cultured human fibroblasts and with epitopes near the LDL receptor binding region of LDL is independent of core lipid composition but changes as a function of LDL size. During the metabolic conversion of VLDL to LDL many subspecies of LDL can be isolated. Chen et al., (1994) have shown conformation differences in apoB among these subspecies of LDL. These investigators found that the accessibility to protease attack in LDL from normolipidemic and hypertriglycerdemic subjects was altered in three peptide regions of apoB. Specifically, apoB in

hypertriglycerdemic LDL exposed more cleavage sites than native LDL. Analysis of the circular dichroic spectra revealed an increase in the β-structure in the hypertriglycerdemic LDL. Binding affinity for the LDL receptor of human fibroblasts decreased markedly with increasing density among hypertriglycerdemic LDL subspecies. These changes were found to be independent of lipid composition but rather varied as a function of LDL size.

Galeano et al., (Galeano N.F., Milne R.W., Marcel Y.L, Walsh M.T., Levy E., Ngu'yen T.D.,Gleeson A., Arad Y., Witte L., Al-Haider M., Rumsey S.C., Deckelbaum R.J., 1994) compared the effect of lipid composition and particle size of triglyceride-rich LDL upon apoprotein B conformation and binding to the LDL receptor. These investigators used three groups of triglyceride-rich LDL and determined apoB conformation by circular dichroism and by analyzing the immunoreactivity of different monoclonal antibodies. Their findings suggested that in triglyceride-rich LDL, the relative content of neutral lipid in the core of LDL in the absence of changes in the size of the particle did not significantly affect apoprotein B conformation or its affinity for the LDL receptor. This would be consistent with LDL particle diameter as being an important determinant of apoB conformation and function.

On the basis of the above observations, it can be seen that heterogeneity in the physical and chemical properties of the LDL modulates apoB conformation and function and subpopulations of particles can be isolated that differ in their apoB circular dichroic spectra (Galeano et al., 1994 and Chen et al., 1994), in their respective reactivities with the LDL receptor (Kleinman et al., 1987, McKeon et al., 1993, Galeano et al., 1994, Chen et al., 1994, Campos et al,. 1996) and with certain anti-apoB monoclonal antibodies (Teng B., Sniderman A., Krauss M.R., Kwiterovich O.P., Milne W.R. and Marcel L.Y., 1985) and in their abilities to activate lipid-modifying enzymes (Liu, M., Krul, E.S. and Subbaiah, P.V., 1992). While it is well accepted that LDL heterogeneity influences apoB

conformation and function, the relative contributions of particle size, lipid composition, surface charge etc. to this modulation remains controversial (Aviram et al. 1988b, Kinoshita et al. 1990, Kunitake et al. 1990, Galeano et al. 1994, Chen et al. 1994). To identify the specific physical and chemical parameters of LDL that influence apoB conformation and function, we have developed a protocol for the preparation of reconstituted LDL particles in which the effects of a single variable can be studied in isolation in a homogeneous population of particles. The ultimate goal of our studies is to reconstitute an apoB-lipid complex which is similar to that found *in vivo*, and this requires progressing from a two component system, apoB-phospholipids, to a more complex system which includes cholesterol and cholesteryl esters. The effect of each bound component on the protein moiety and on the relative amounts of the other naturally occurring bound lipids can then be investigated in a systematic manner. Here we describe the physicochemical and immunochemical properties of partially delipidated apoB and apoB that has been reconsitituted with phospholipid.

1.6 Specific Aims:

1. To develop a novel method for the preparation of biologically active low density lipoproteins.
2. To elucidate the effect of specific lipids on LDL assembly.
3. To determine the electrokinetic, physicochemical and immunochemical properties of reconstituted LDL particles.

Experimental Procedures

2.1 Materials:

1-palmitoyl-2-oleoyl phosphatidylcholine, dimyristoyl phosphatidylcholine and guanidine HCl were obtained form Avanti Polar Lipids (Birmingham, AL) and Bethesda Research Laboratories (Bethesda, MD). All other reagents were analytical grade.

Methods:

2.2 Isolation of LDL:

LDL was isolated from fresh plasma of normolipidemic donors by sequential ultracentrifugation (Schumaker V.N., and Puppione D.L., 1986). A mixture of preservatives was added to the freshly drawn blood; final concentrations were 500 units/ml penicillin-G, 50 μg/ml streptomycin sulfate, 0.05% EDTA , 0.05% NaN_3, 0.2 mM phenylmethylsulfonyl fluoride, and 0.005% 4-methyl-2,6-tert-butyl phenol. The plasma was distributed among eight screw top, thick walled polycarbonate bottles of a 55.2 Ti rotor. The plasma was centrifuged at 8°C and 40000 rpm for 28 h. The top layer of tubes contained VLDL, while the other plasma proteins and lipoproteins sunk toward the bottom. The VLDL were removed and approximately 8 ml of the intermediate, clear layer was saved for density determination. The remaining infranatent solution was used to resuspend the protein pellet at the bottom of each tube using a glass stirring rod. After the pellet was resuspended, the infranatent solutions were pooled and the appropriate amount of concentrated KBr solution was added, together with water to adjust the density to 1.019 g/ml. This solution was divided between 6 centrifuge tubes and centrifuged at 40000 rpm, 8°C for 28 h to float the IDL to the top. The IDL were removed .and the clear zone was saved for density determination, and an appropriate volume of concentrated KBr and water were mixed with the combined infranatent solutions to adjust the density to 1.063 g/ml. The solution was divided between 6 tubes and

centrifuged at 40000 rpm , 8°C, for 28 h to float LDL to the top. The LDL were removed in small volumes with a capillary pipet. The LDL containing fractions were visible yellow and optically clear. The LDL were dialyzed four times for 4-12 h against 50 mM NaCl-50 mM Na_2CO_3 (pH 10).

2.3 Characterization of LDL:

2.3.1 Composition:

Total cholesterol, free cholesterol and triglycerides and phospholipids were determined enzymatically using Boehringer Mannheim kits and manufacturer's suggested procedures. Free cholesterol assays were based upon the oxidation of cholesterol with cholesterol oxidase to produce hydrogen peroxide, which is further reacted with catalase and methanol to produce formaldehyde. A final colormetric reaction occurs when formaldehyde is reacted with ammonium ions and acetylacetone to produce a yellow colored compound 3,5-diacyetyl-1,4-dihydrolutindine(Allain C.C., Poon L.S., Chan C.S. and Richmond W., 1974). Total cholesterol values were determined after cleaving fatty acids with cholesterol esterase and esterified cholesterol was then determined mathematically by subtracting unesterified from total cholesterol values. Triglyceride assays were based upon lipase hydrolysis of triglyceride to glycerol, subsequent action by glycerol kinase and glycerol phosphate oxidase to produce hydrogen peroxide, and a final colormetric reaction with peroxidase, 4-aminophenazone and 4-chlorophenol to produce a pink colored compound, 4-(p-benzoquinone-mono-imino)-phenazone (Bucolo G. and David H. 1973). Phospholipids were based on the reaction of phospholipids with phospholipase B to produce choline and phosphatidic acids. The choline was further reacted with choline oxidase to produce betaine and hydrogen peroxide, and a final colormetric reaction with peroxidase, 4-aminophenazone and phenol to produce a pink colored compound, 4-(p-benzoquinone-

mono-imino)-phenazone (Trinder, P., 1969). Proteins were determined by modified Markwell Lowry method (Markwell M.K., Hass S.M., Bieber L.L., and Tolber N.E., 1978)

2.3.2 Charge and Mobility:

Electrophoretic mobilities (U) of proteins and lipoproteins were determined by electrophoresis on preformed 0.5% agarose gels (Beckman, Paragon Lipoprotein Electrophoretic kit). Samples (6 μg protein) were applied to gel wells and allowed to penetrate into the gel for 5 min before the electric field was applied. A Bio-Rad model 702 power supply was used to apply a voltage of 100 volts across a gel distance of 5.5 cm. Electrophoresis was continued for 30 min at 25°C in the kit barbital buffer (pH 8.6, 0.05 ionic strength). After electrophoresis, the gels were fixed in a solution of ethanol-acetic acid-water (60:10:30) (v/v/v), oven dried (80^{0}C for 1 h) and then stained (5 min) with a 0.15% Coomassie Blue R250 solution. Gels were destained in a solution of methanol-acetic acid-water 35:25:40 (v/v/v) for about 10 min or until the background adjacent to protein or lipoprotein bands was clear and the stain intensity of the bands was uniform. The migration distance was measured directly from the stained gel and was the distance (=0.5 mm) from the point of loading to the center of each stained band. Electrophoretic migration patterns observed by staining the protein moiety of each lipoprotein were essentially identical to those observed when the lipid was stained with Sudan Black B. Apo A-I were run on each gel as internal standards to correct for slight gel to gel variations in electrophoretic mobility. Electrokinetic theory was applied to estimate colloidal particle net charge and charge density from electrophoretic mobility (Abramson, H.A., L.S Moyer, and M.H, Gorin, 1942)

2.3.3 Size:

The apparent molecular weight of apoB was determined by SDS gel electrophoresis on 5% polyacrylamide 0.75mm gels, pH 8.8, in the presence of beta-meracaptoethanol, with a 4% acrylamide, pH 6.8, stacking gel. The running buffer was 25 mM Tris-glycine, pH 8.3, containing 0.1% SDS and reference standards used were: phosphorylase b (Mr 92 500), bovine serum albumin (Mr 66 200), ovalabumin (Mr 45 000), carbonic anahydrase (Mr 31 000), soyabean trypsin inhibitor (Mr 21500), and lysozyme (Mr 14 400). Proteins were stained with 0.25% Coomassie R250 in acetic acid/methanol/H_2O (1:4.5:4.5) and destained in acetic acid/methanol/H_20 (1:4.5:4.5).

The homogeneity and hydrodynamic diameters were estimated by nondenaturing gradient gel electrophoresis using precast 4-15% polyacrylamide gels using the Phast system (Pharmacia Biotech Inc.) and reference globular proteins (17.0 nm thyroglobulin, 12.2 nm ferritin, 10.4 nm catalase, 8.2 nm lactate dehydrogenase, and 7.1 nm albumin) provided by a high molecular weight calibration kit (Pharamacia, Uppsal) as described (Williams P.T., Krauss R.M., Nichols A.V., Vranizan K.M., and Wood P.D., 1990)

2.4 Preparation of Reconstituted LDL:

2.4.1 Delipidation of LDL:

The apoB from LDL was isolated using a delipidation procedure essentially by the method developed by Helenius and Simons (Helenius A. and Simons K., 1971). LDL was dialyzed against 50 mM NaCl-50 mM Na_2CO_3 (pH 10) overnight. This native LDL was incubated with sodium deoxycholate (NaDOC) at a weight ratio of 1:12 (apoB:cholate) for 30 min. in the dark. After 30 min. incubation at room temperature, the solubilized protein was separated from lipid and detergent

by gel filtration on a Sepharose CL-4B column (1.6cm×60cm). The column was equilibrated and eluted with 50 mM sodium chloride-50 mM sodium carbonate-10 mM NaDOC (pH=10) at 4°C. Three milliliter fractions were collected and analyzed for protein, cholesterol and phospholipid. The protein peaks were concentrated using centriprep concentrators. To remove detergent, the concentrated sample was passed through a Sephadex G-75 column (1.6×30 cm) and eluted with 0.01 M Tris-HCL buffer, pH=9. Three milliliter fractions were collected, the protein eluted in the void volume and the bile salt eluted close to the total volume of the column. The protein peak was further concentrated using a centriprep concentrator. To determine the amount of detergent associated with apoB after gel filtration various amounts of sodium deoxycholate were incubated with apoB and the mixtures were electrophoresed on 0.5% agarose gels. A relationship was observed between apoB surface charge and concentration of sodium deoxycholate, from which we estimated the isolated apoB to have less than 0.04 molecules of sodium deoxycholate per molecule of protein.

2.4.2 Assessment ofApoB aggregation:

Immulon II Removawells (Dynatech Laboratories, Chantilly, VA) were coated by an overnight incubation with 100 µl of anti-human apoB monoclonal antibody 1D1 (5 µg/ml, 5 mM glycine, pH 9.2) and subsequently saturated by incubations for 1 h with 100 µl of 1% bovine serum albumin-PBS, pH 7.4. Serial dilution of test and control apoB containing samples (4 µg/ml) were prepared in microtiter plates and added to the plates and incubated for 3 h. The plates were washed four times with wash buffer (0.15 M NaCl containing 0.025% Tween 20). ^{125}Iodine-labeled anti-human apoB monclonal antibodies (100 µl) 1D1, 2D8 and 3F5 (1/100 dilution) (10^4 cpm/ng) (10 µg/ml) were added to the 1D1-apoB captured mixtures and incubated for 4 h and washed with Tween-saline

solution as above and counted for bound radioactivity (Raffai R., Maurice R., Weisgraber K., Innerarity T., Wang X., MacKenzie R., Hirama T., Watson D., Rassart E., and Milne R., 1995)

2.5 Reconstitution:

2.5.1 Apo B reconstitution using dimyristoyl phosphatidylcholine (DMPC) and palmitolyeol phosphatidylcholine (POPC):

The ability of apoB to associate with DMPC and POPC was determined by a turbidity clearance assay. The desired amount of DMPC was dried to completion under nitrogen and solubilized in Tris buffer (1 mg/ml). Before the assay, the protein and lipid sample (DMPC/protein molar ratio=100-300/1) were preincubated independently at 24°C for 10 min.. The rate of lipid-protein association was then followed by monitoring the reduction in turbidity at 325 nm. Similar experiments were performed at temperatures ranging from 4°C to 24°C with POPC. The reconstituted particles were isolated using size exclusion chromatography on a Superose-6 column.

2.6 Conformation Characterization of rLpB:

2.6.1 Circular Dichroism (CD):

The average secondary structures of apoB were monitored by CD spectroscopy on a Jasco J41A spectropolarimeter calibrated with a 0.1% (w/v) D-10-camphorsulfonic acid solution. CD spectra were measured at 24°C at a 0.1-cm path length quartz cell, and eight scans from 260 to 184 nm were collected and averaged. The percent α-helix in apoB was calculated from the molar ellipticity at 222 nm using a mean residue weight of 112.9. The effect of GdnHCl concentration on the secondary structure of apoB and native LDL was monitored by the changes in molar ellipticity at 222 nm. Aliquots of each complex (33 μg of protein/ml of buffer) were incubated with 0-6 M GdnHCl in 0.l05 M phosphate buffer, pH 7.2, for 72 h at 4°C. The free energy of unfolding of the

isolated apoB and apoB present on LDL was calculated as described previously assuming the protein to unfold in a cooperative two state manner (Sparks, D.L., Lund-Katz, S., and Phillips, M.C, 1992).

2.6.2 Immunoreactivity of rLpB with monoclonal antibodies:

Immulon II Removawells (Dynatech Laboratories, Chantilly, VA) were coated by an overnight incubation with 200 µl of reference LDL (30 µg/ml, 5 mM glycine, pH 9.2) and subsequently saturated by incubations for 1h with 250 µl of 1% bovine serum albumin-PBS, pH 7.4. Serial dilutions (125 µl) of test and control LDL were prepared in microtiter plates. 125 µl of mAb, appropriately diluted in 1% bovine serum albumin-PBS, was added to the diluted LDL and allowed to incubate for 4 h at room temperature. Aliquots (200 µl) of the LDL-mAb mixture were transferred to the LDL-coated Removawells that had been washed with a solution of 0.15 M NaCl containing 0.025% Tween 20. The wells were incubated overnight and again washed with the Tween-saline solution as above and counted for bound radioactivity (Milne R.W., Philip K.W., and Marcel Y.L., 1992). Five mAbs mapping different epitopes of the apoB molecule were used: 1D1, 2D8, 3F5, 4G3 and 5E11, which react between residues 474-539, 1438-1480, 2922-2980, 2980-3084 and 3441-3687 respectively (Pease et al,1990).

2.7 Mathematical Analysis:

Significance of difference between population means for the control group and experimental group were determined by an unpaired Student's t-test. Correlation coefficients were determined by linear regression analysis .

Results

3.1 ApoB isolation and characterization:

3.1.1 ApoB isolation by delipidation of LDL:

LDL lipids were separated from apoB by combining with NaDOC and then by chromatography of the clear yellow solution on a Sepharose CL-4B column, equilibrated with 35 mM NaDOC-0.05 M sodium carbonate (pH 10). The protein eluted in the void volume as an optically clear solution with a recovery of 74% (mean of 12 experiments). In order to remove any excess bile salt, the apoprotein was applied to a Sephadex G-75 gel filtration column. The protein eluted in the void volume as a clear solution with a recovery of 85% (mean of 12 experiments). This aqueous soluble apoB contained no detectable amounts of phospholipids, cholesterol and cholesteryl esters but had retained ~70% of the triglycerides from LDL (Table 1 (representative example)). SDS-polyacrylamide electrophoresis of the protein showed no apoB degradation and revealed an apparent molecular weight of approximately 500,000 daltons (Figure 4). Delipidation of LDL at concentrations greater than 3-4 mg LDL protein/ml yielded apoB-TG preparations that still contained cholesterols and phospholipids.

Table 1: Typical molar lipid compositions of native and reconstituted lipoprotein particles

Complex	Phospholipid initial[a]	Phospholipid final[b]	Free Cholesterol	Cholesteryl Ester	Triglyceride
			mol/mol apoB		
Native LDL	509	-	750	634	367
ApoB-TG[c]	-	-	-	-	242
100-POPC-apoB	100	75	-	-	242
300-POPC-apoB	300	265	-	-	242

[a] Phospholipid content before reisolation on a Superose-6 gel filtration column.
[b] Phospholipid content after reisolation on a Superose-6 gel filtration column.
[c] ApoB associated with triglycerides

3.1.2 Assessment of ApoB Aggregation:

ApoB aggregation was determined through a sandwich assay using immobilized 1D1 as a capture antibody. LDL or apoB-TG were added to 1D1-coated microtiter wells and, after a 3 h incubation, bound apoB was detected with either ^{125}I-1D1, ^{125}I-2D8 or ^{125}I-3F5. We have previously shown that neither 2D8 nor 3F5 compete with 1D1 for binding to LDL (Milne et al., 1983). Figure 5 shows that little ^{125}I-1D1 binds to 1D1-captured LDL or apoB-TG which is consistent with only one 1D1 epitope being present per LDL or apoB-TG particle. As expected, ^{125}I-2D8 and ^{125}I-3F5 bind well to 1D1-captured LDL or apoB-TG. These results indicate that aggregation is minimal in the apoB-TG.

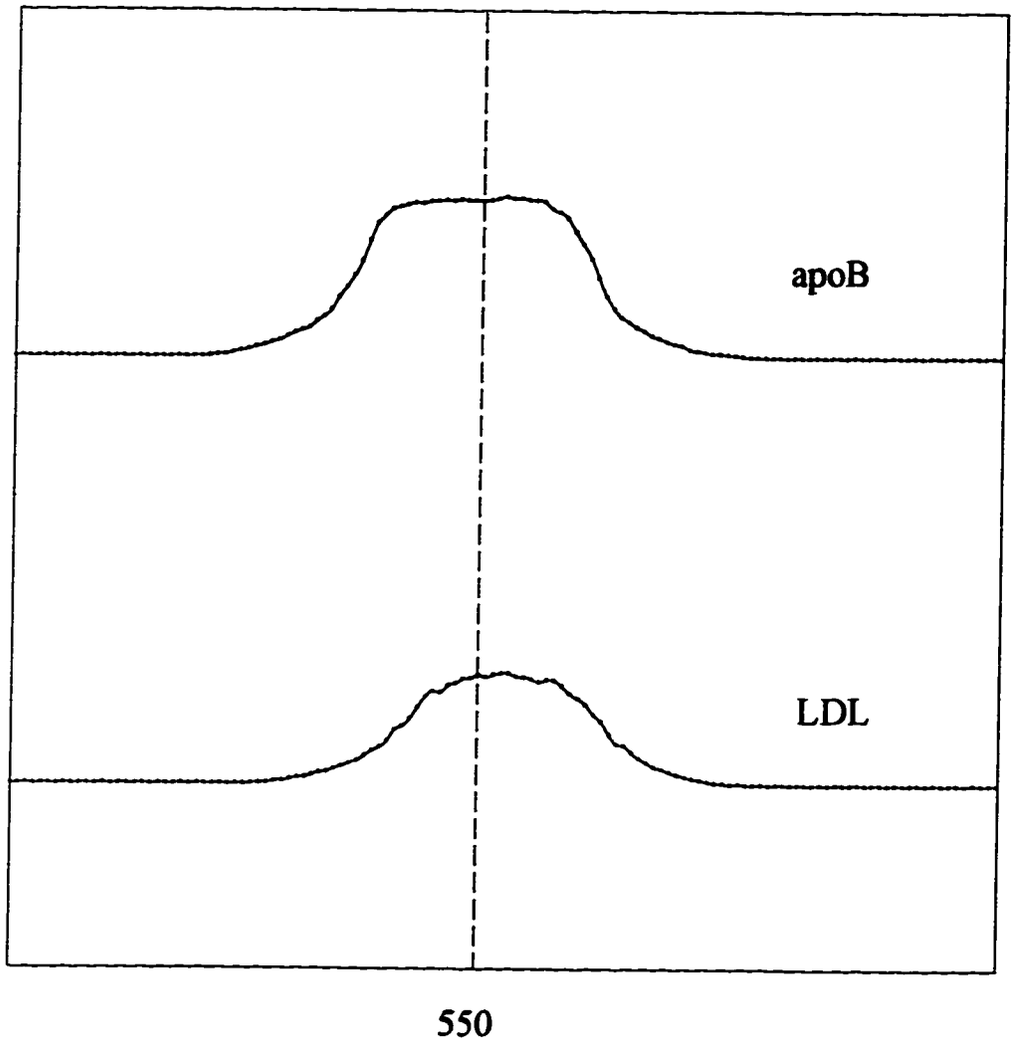

Figure 4: **SDS polyacryalamide gel electrophoresis**. Densiometeric profiles of partially delipidated apoB (apoB) and native LDL (LDL) subjected to 7% SDS polyacrylamide gel electrophoresis are shown. Estimated molecular weights were determined from reference standards as described in the experimental procedure.

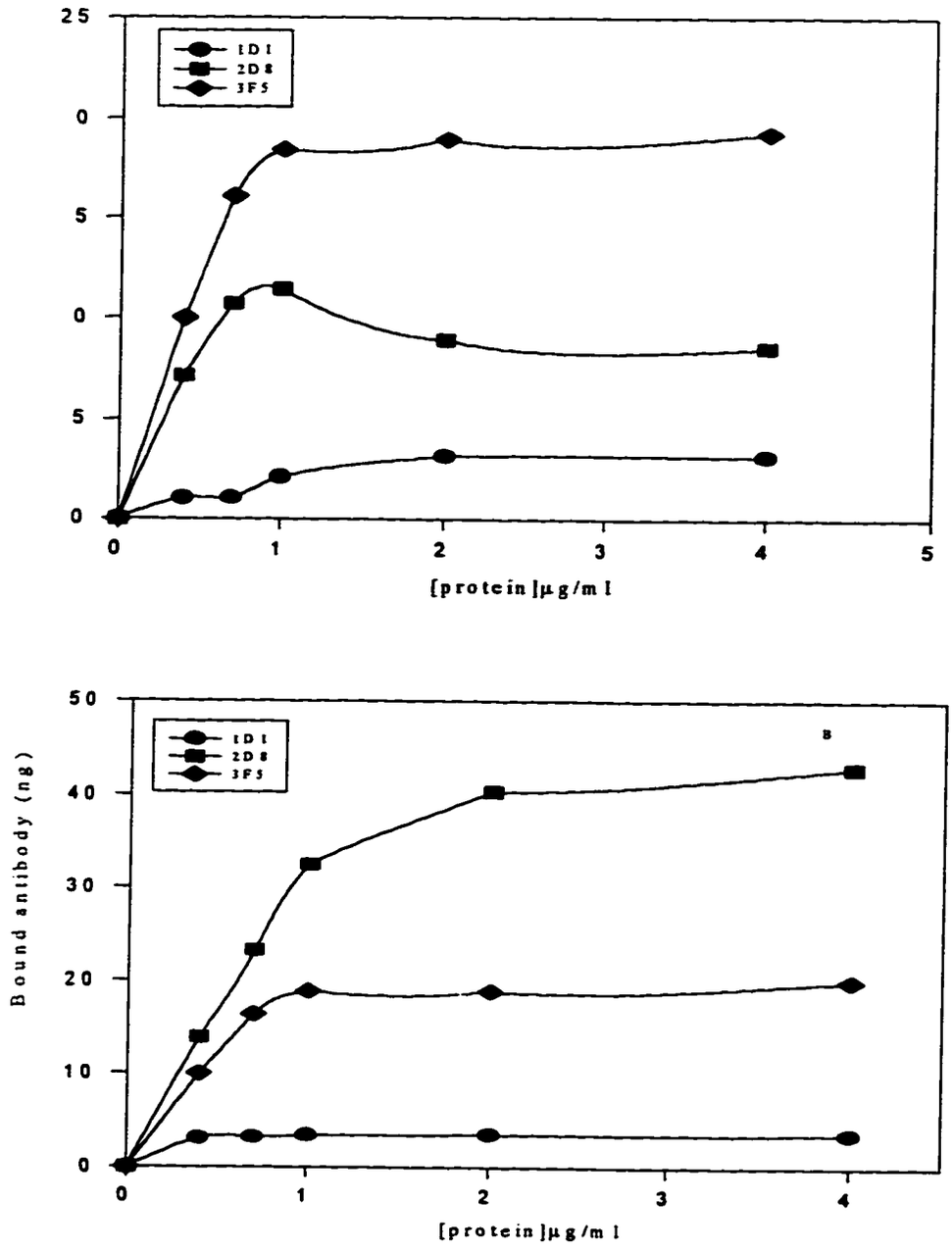

Figure 5 . **Sandwich radioimmunoassay**. Immulon II removawells were coated by an overnight incubation with monoclonal antibody 1D1 (5 µg/ml, 5 mM glycine, pH 9.2) and subsequently saturated with 1% bovine serum albumin-PBS, pH 7.4. Serial dilutions of test and control apoB containing samples (4 µg/ml) were prepared in microtiter plates and added to the plates. Iodine labeled antibodies (10µg/ml) 1D1, 2D8 and 3F5 (1/100 dilution) were added to the 1D1-apoB captured mixtures and counted for bound radioactivity. Panel A depicts the immunoreactivity of the various antibodies with apoB-TG while Panel B shows the immunoreactivity with LDL.

3.2 ApoB complexation with phospholipids and characterization:

3.2.1 Reconstitution of phospholipids (POPC & DMPC) with apoB:

Various amounts of either POPC or DMPC were incubated in the presence of TBS buffer and vortexed vigorously; the obtained mixture was turbid. ApoB-TG was combined at various molar ratios with POPC vesicles and the kinetics of association were monitored by determining the reduction in turbidity at 325 nm. As illustrated in Figure 6, apoB-TG was able to solubilize POPC vesicles within the first 10 min. of incubation; the reaction was rapid and spontaneous. This association occurred with similar rapidity at various temperatures, above and below the transition temperature of POPC. Over a reaction time of 30 min. a sigmoidal shaped curve was observed, which was composed of a constant initial absorbance, followed by a rapid decrease in absorbance between t=3-10 and then a plateau with no further change in absorbance. Similar results were obtained when DMPC was substituted for POPC. In order to compare the ability of other apoproteins to interact with lipid vesicles relative to apoB we also examined the kinetics of association of POPC and DMPC with the exchangeable apolipoprotein apoA-I. Similar to previous studies by Pownall et al., (Pownall J. Henry, Pao Q., Hickson D., Sparrow J.T., Kusserow S., Massey J.B., 1981), our results showed that apoA-I did not interact with POPC vesicles but gradually solubilized DMPC to yield an exponential clearance curve with first order kinetics. After the complexation of apoB and POPC vesicles, the lipid:protein complexes were separated from free lipids by chromatography on a superose-6 gel filtration column. Examination of the size of the isolated particles by 4-15% gradient gel electrophoresis showed the rLpB particles to exhibit a single discrete band (Figure 7).

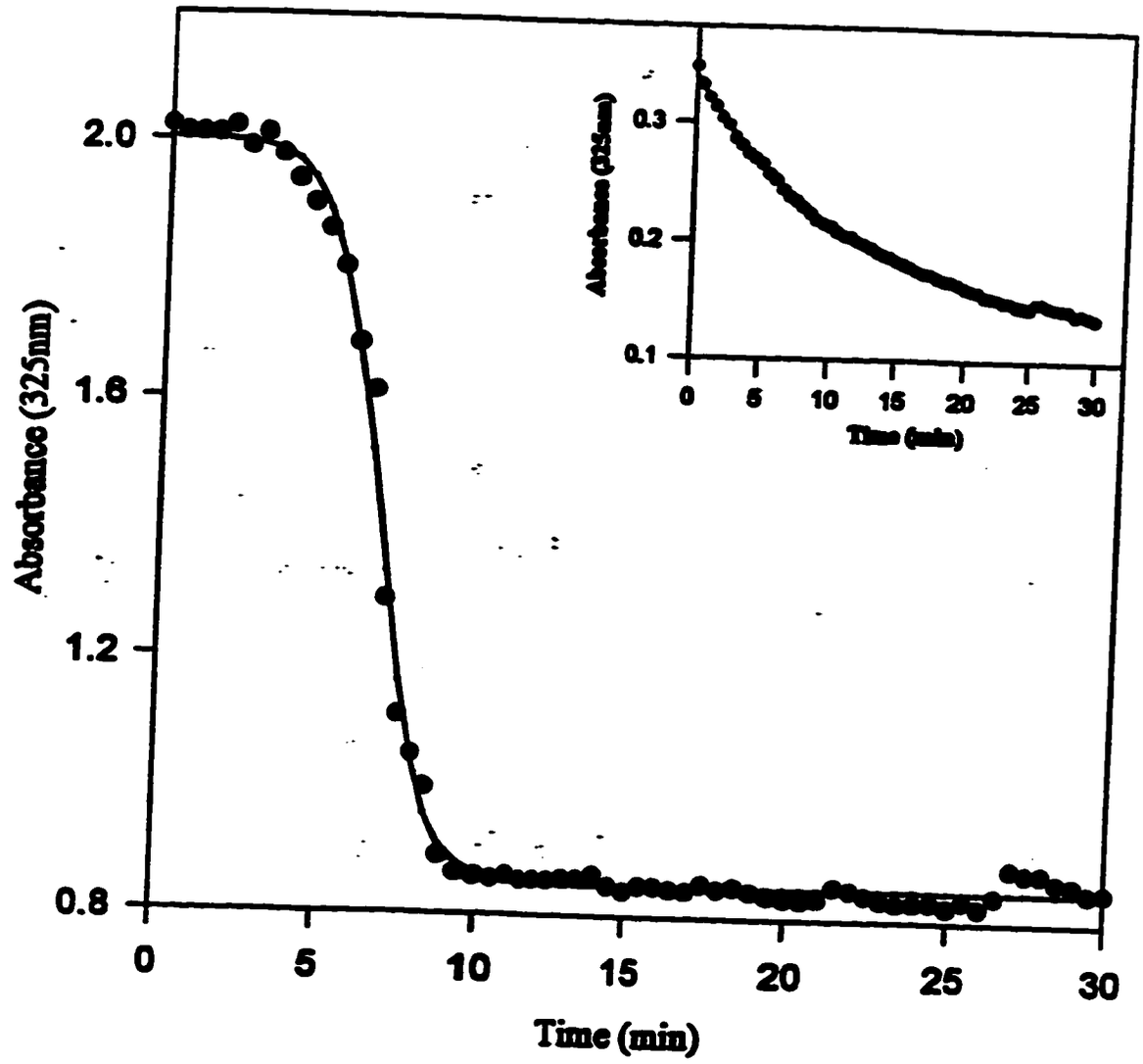

Figure 6. **Kinetics of POPC association with apoB.** The reduction in turbidity (absorbance at 325 nm) at 10°C of POPC vesicles (1 mg/ml Tris Buffer) after the addition of partially delipidated apoB is shown. The inset shows the reduction in turbidity at 24°C of DMPC vesicles (1 mg/ml Tris Buffer) after addition of apoA-I.

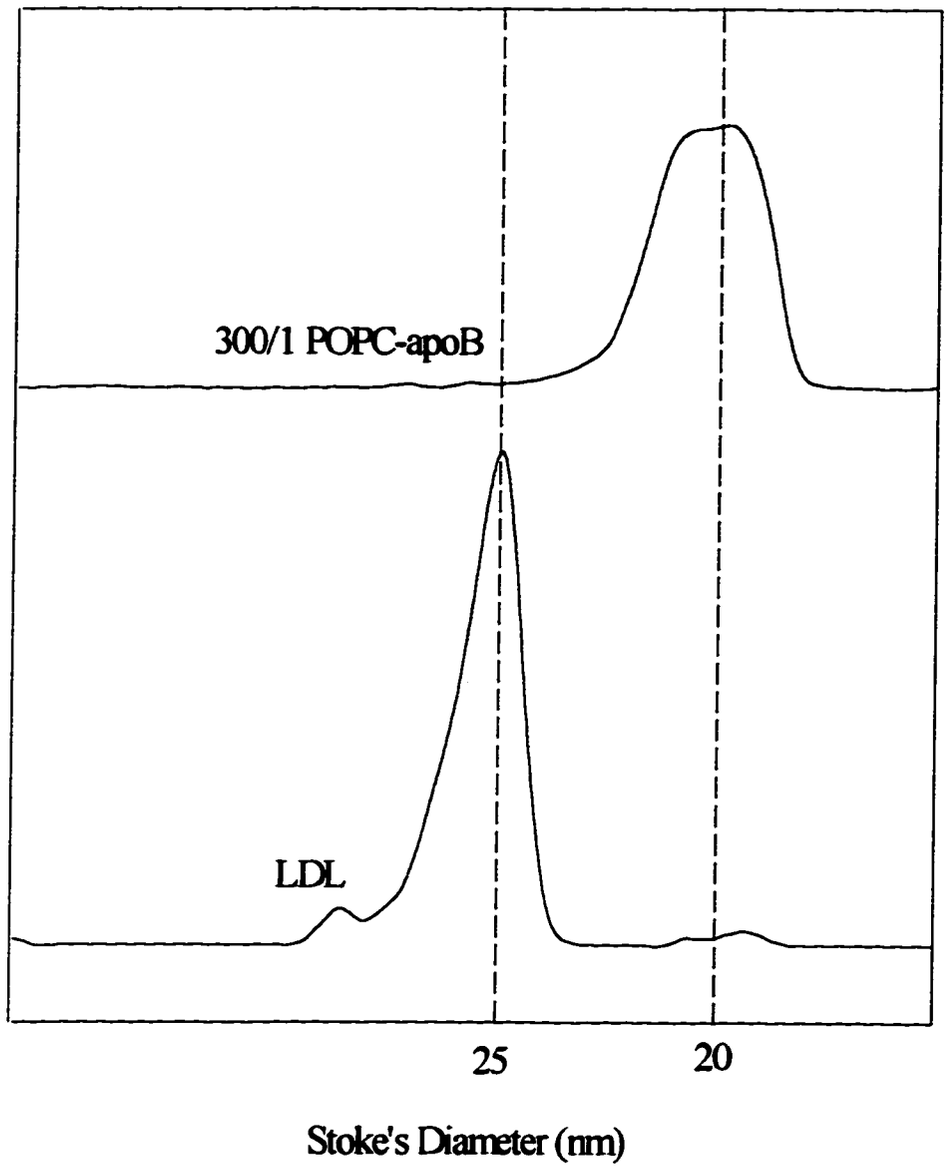

Figure 7: **Nondenaturing gradient gel electrophoresis**. Densitometeric profiles of rLpB (300/1 POPC-apoB) and native LDL subjected to 4-15% nondenaturing gradient gel electrophoresis are shown. Estimated hydrodynamic diameters were determined from reference standards as described in the experimental procedure.

3.2.2 Characterization of the secondary structure and stability of apoB in nLDL and rLpB particles:

The far UV CD spectra for LDL, reconstituted particles (rLpB) and apoB showed a shallow negative trough between 210 and 220 nm, which indicates that apoB contains a small but significant amount of α-helical structure (Figure 8 and Table 2). The addition of 300 molecules of POPC per mole apoB increased the amount of α-helix in apoB and yielded a spectrum that is very similar to that for LDL. This suggests that much of apoB secondary structure is similar on the two different lipoprotein complexes. The data is also consistent with studies from other labs, which have shown that apoB

Table 2: Secondary structure and stability of free and lipid-bound apoB

Complex	$D^{a}_{1/2}$ M GdnHCl	α-helix[b] %
Native LDL	2.52	49.1
ApoB-TG[c]	2.89	17.0
100-POPC-apoB	2.59	27.5
300-POPC-apoB	2.11	34.2

[a] Midpoint of GdnHCl denaturation ± 0.03M (S.D.).
[b] α-helix content determined from molar ellipticities at 222 nm ±4% (S.D.).
[c] ApoB associated with triglycerides

undergoes a structural change when complexed with lipid, wherein its α-helix content increases (21). The general features of the CD spectra of particles prepared from 100 and 300 molecules of POPC were similar to those observed on LDL, except for the slight leftward shift in spectra for LDL relative to rLpB and apoB. This leftward shift in spectra for LDL may be due to the contributions of

cholesterol and cholesteryl esters to the region below 220 nm, which are absent in the rLpB (Walsh M.T., 1983).

Isothermal denaturation of LDL and rLpB were performed and are illustrated in Figure 9. In the presence of GdnHCl, apoB on LDL undergoes a gradual unfolding but appears to be resistant to complete denaturation. The protein exhibits a complex multi state unfolding profile and has at least two partially stable intermediates. At the highest concentration of GdnHCl, LDL-bound apoB retains much of its secondary structure and still has approximately 85% of its amphipathic α-helices. Delipidated apoB-TG unfolds in a manner which parallels the curve obtained for LDL but commencing and terminating at a much lower α-helical content. Unlike LDL, the α-helices of lipid-poor apoB are completely unfolded and in random coil in the presence of 5.8 M GdnHCl. Phospholipidation of apoB appears to stabilize and protect apoB secondary structure to denaturation by GdnHCl. The addition of 100 molecules of POPC to apoB yields a denaturation curve that is similar and parallel to lipid-poor apoB, but that has a consistent increase in α-helicity and does not result in the complete unfolding of apoB. Addition of 300 molecules of POPC significantly increases the overall α-helical content of apoB and as with LDL, the effectiveness of GdnHCl in unfolding apoB is also significantly decreased. Increasing the concentration of GdnHCl to 5.8 M gave rise to only a small reduction (30%) in apoB α-helicity for the 300 POPC particle.

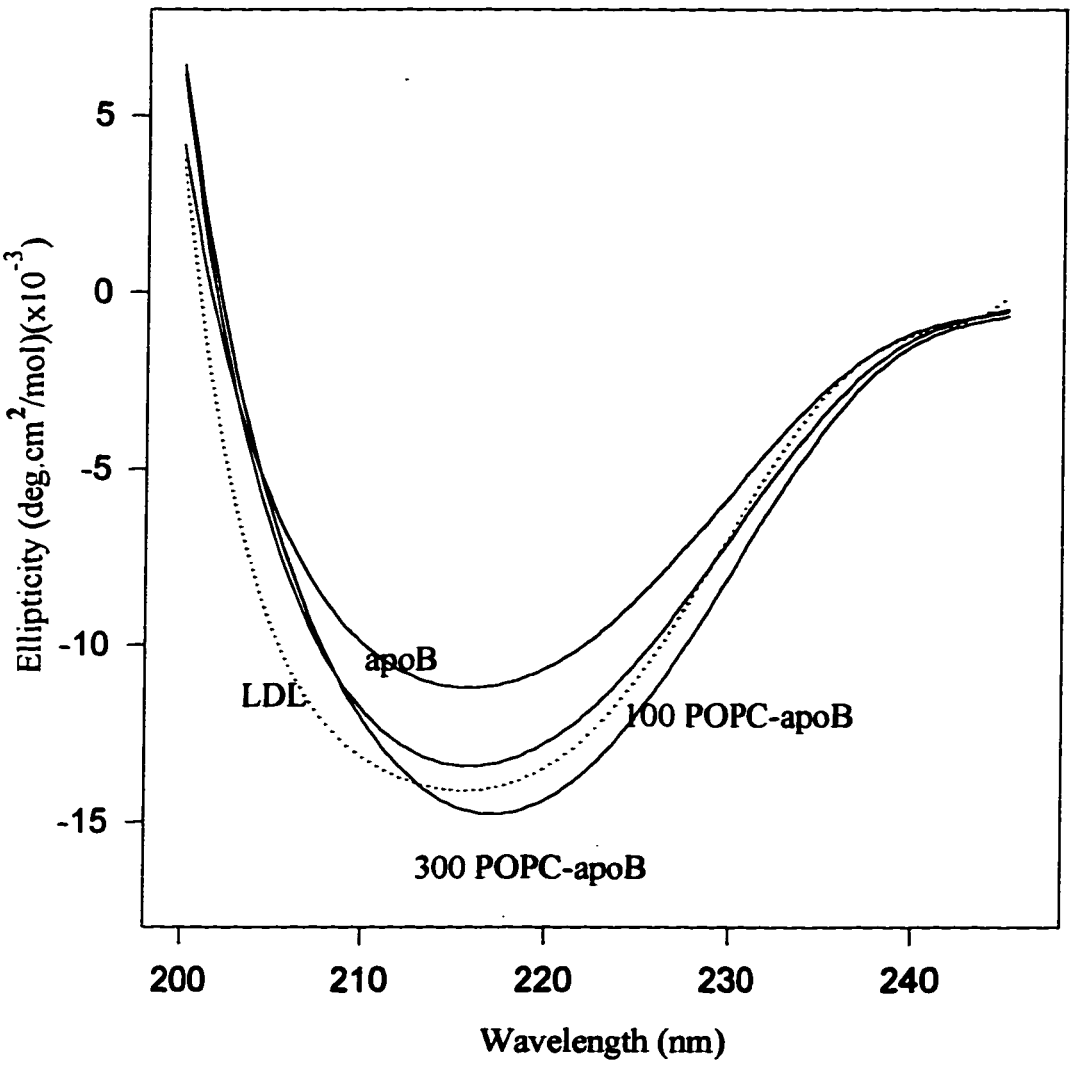

Figure 8: **Circular dichroic spectra of apoB on different LpB particles**. The spectra of apoB on native LDL (dashed line) were recorded from 260 to 200 nm with a Jasco 41A spectropolarimeter and are shown relative to the spectra for apoB-TG and rLpB particles containing 300 and 100 molecules of POPC (solid lines).

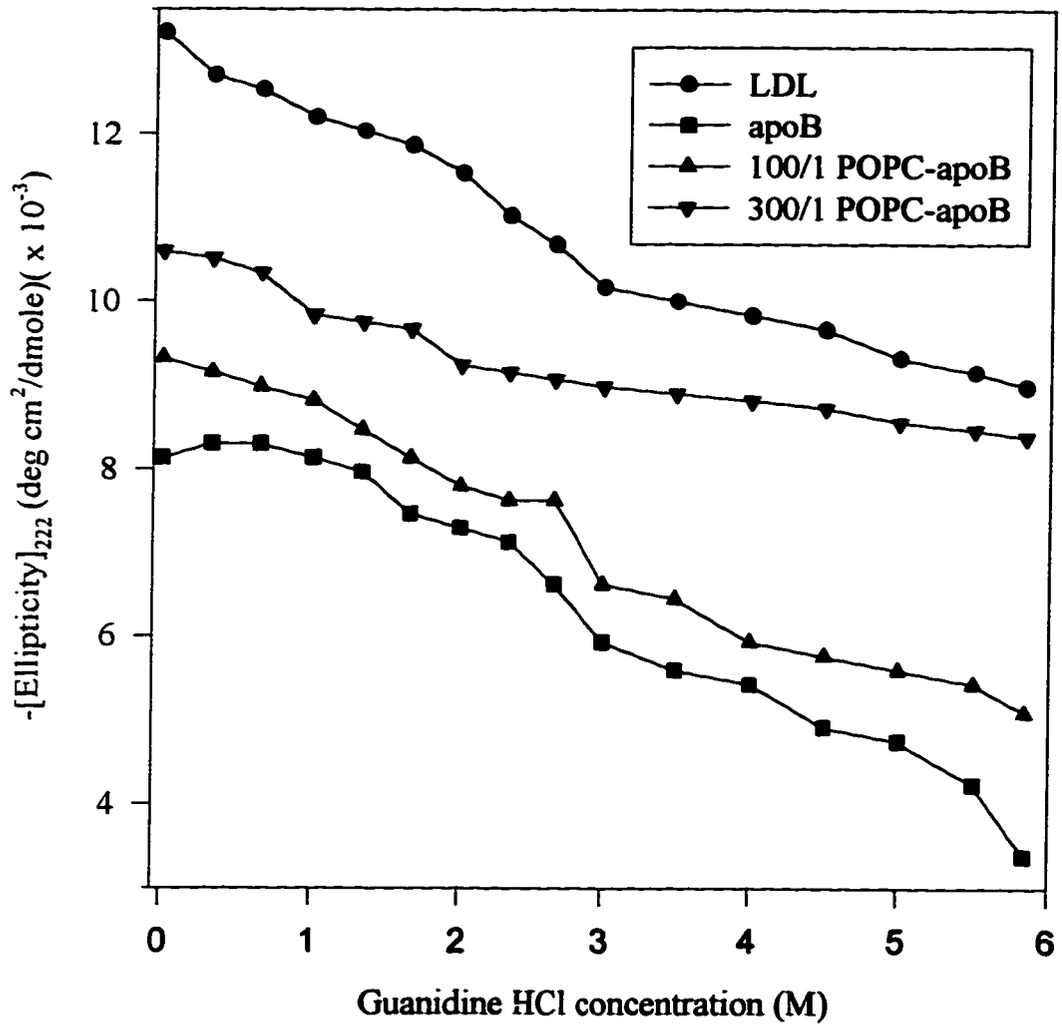

Figure 9: **Effect of GdnHCl concentration on the molar ellipticity at 222 nm for LDL and rLpB.** Aliquots of each protein (33.3 μg/ml) were preincubated with 0 to 6 M GdnHCl in 0.05 M phosphate, buffer for 72 h at +4°C. The molar ellipticity at 222 nm was determined for LDL, apoB and rLpB particles containing 100 and 300 molecules of POPC. Values are the average of duplicate determinations.

Table 3: Typical electrophoretic characteristics of native and reconstituted lipoprotein particles

Complex	Mobility[b] (-) um $s^{-1}cm\ V^{-1}$	Surface Potentials[c] (-) (mV)	Charge Density[d] (-) ($x10^2$ esu/cm^2)	Valence[e] (-e)
Native LDL	0.311	6.0	6.48	82.1
ApoB-TG[f]	0.150	2.9	3.12	39.5
100-POPC-apoB	0.185	3.6	3.85	48.8
300-POPC-apoB	0.251	4.9	5.22	66.2

[b] Corrected electrophoretic mobility (0.5% agarose)± 0.01 (S.D).
[c] Charge potential at the particle surface± 0.2 (S.D.).
[d] Net density of surface charge ± 0.02 $x10^2$ (S.D.). esu, electrostatic units.
[e] The number of excess negative charges in electronic units ±0.1 (S.D.).
[f] ApoB associated with triglycerides.

3.2.3 Characterization of the net charge of apoB and rLpB complexes:

Electrokinetic properties of LDL and rLpB were assessed partially by agarose gel electrophoresis as illustrated in Table 3 (representative example). On agarose gel electrophoresis, LDL exhibits a distinct band that migrates into a region of β-mobility with a surface potential of -6 mV (Figure 10). ApoB alone exhibits a band located near the origin of the gel with a surface potential of -2.9 mV. The addition of POPC to apoB resulted in an increase in mobility, whereby 300 molecules of POPC yielded a particle with a mobility similar to that observed on LDL. Electrokinetic analysis of the electrophoretic mobilities revealed an increase in the magnitude of charge densities and valence with increasing phospholipid content. ApoB-TG has a lower charge density relative to POPC-apoB and LDL. Therefore, the addition of phospholipids changes the conformation of apoB in a manner that exposes more negative charges and an overall valence that is approximately 81% that of apoB on LDL.

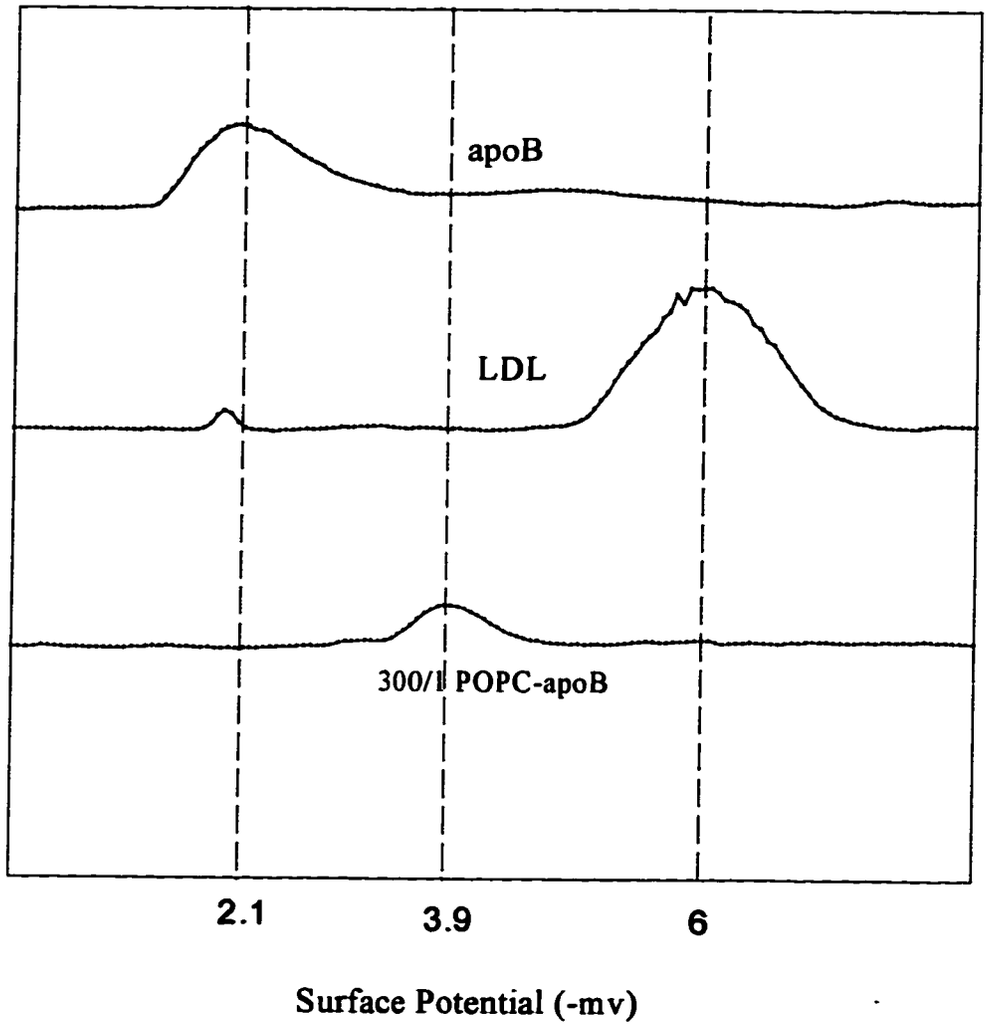

Figure 10: **Agarose gel electrophoresis.** Densitometric profile of rLpB (300/1 POPC-apoB), partially delipidated apoB (apoB) and native LDL (LDL) subjected to electrophoresis on 0.5% agarose gels are shown. Estimated surface potentials were determined from reference standands as described in the experimental procedure.

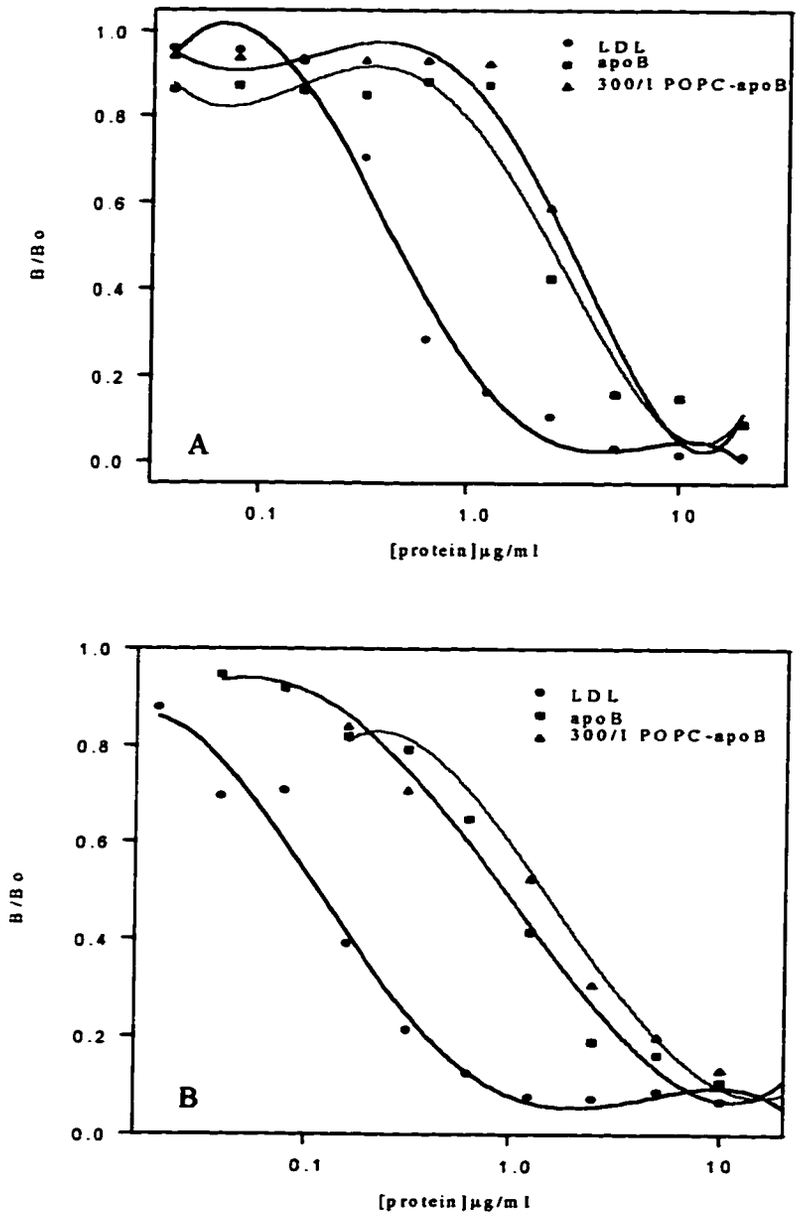

Figure 11: **Competitive radioimmunoassay of apoB, rLpB and LDL with various mAbs.** Solid phase radioimmunoassay were performed, mAb 1D1, 2D8, 3F5, 4G3 and 5E11 appropriately diluted in bovine serum albumin-PBS solution were incubated overnight with dilutions of competing ligand. Binding is expressed as the ratio of ^{125}I labeled antimouse Ig bound in the presence of the added competing antigen (B), 1D1 (panel A) or 3F5 (panel B) to ^{125}I labeled antimouse Ig bound in the absence of competing antigen (Bo).

3.2.4 Immunoreactivity of apoB on LDL and rLpB with monoclonal antibodies:

The immunoreactivities of rLpB and apoB-TG were examined with mAbs, 1D1, 2D8, 3F5, 4G3 and 5E11. Competition curves for two of the mAbs, 1D1 and 3F5 are shown in Figure 11 and results for all of the mAbs are summarized in Figure 12. The competition curves for the apoB-TG and the rLpB obtained with mAbs 1D1, 2D8, 3F5, 4G3 and 5E11 were parallel to those of native LDL. The immunoreactivity (ED_{50}) of the particles were calculated as the concentration of protein necessary for 50% displacement of the maximum binding of the monoclonal antibody to the immobilized control LDL (Figure 12). For all of the mAbs, the ED_{50}s for apoB-TG were about five fold higher than for LDL. Inclusion of 300 molecules of POPC to apoB gave rise to a significant reduction in ED_{50} for the antibodies 2D8 and 4G3, but had no major affect on ED_{50} values for the other monoclonal antibodies studied. Incorporation of POPC into apoB appeared to bring about structural changes in specific domains in apoB, but did not completely regenerate the immunoreactivity of apoB as detected by this panel of mAbs.

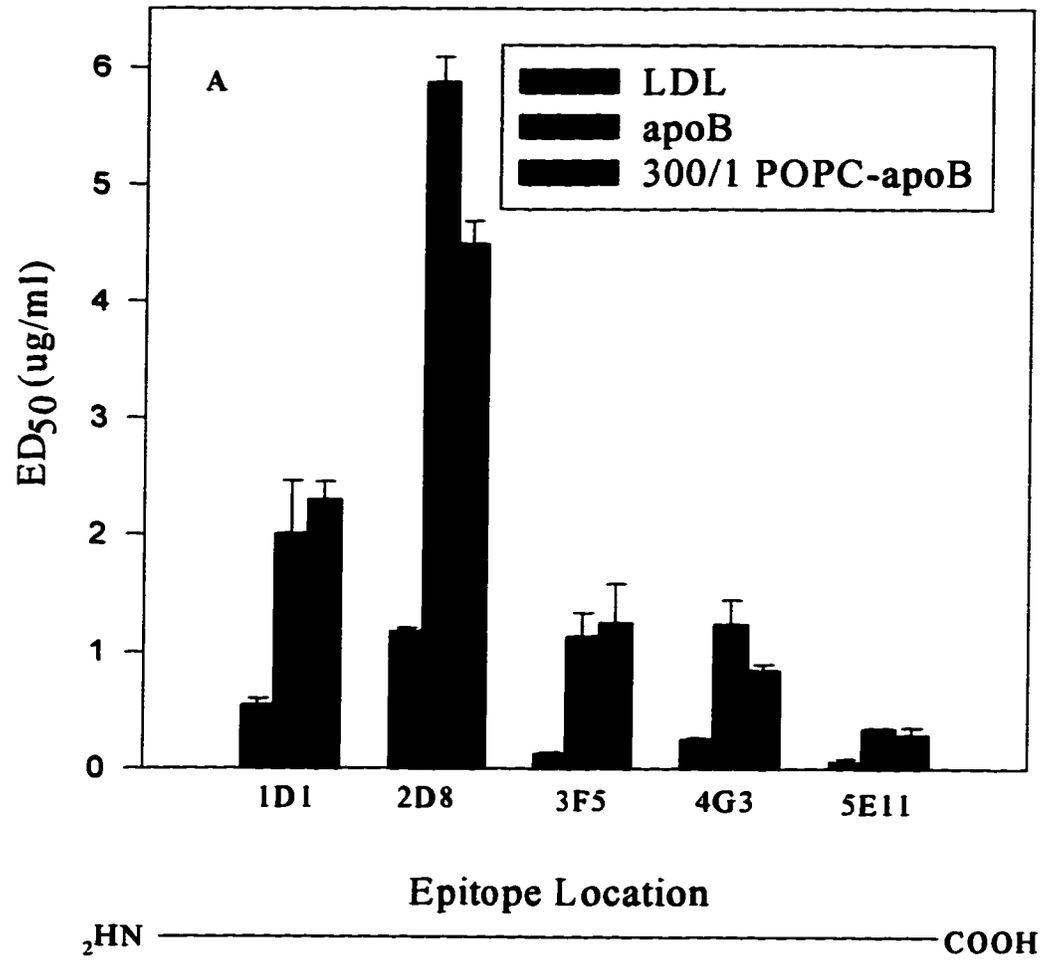

Figure 12: **Immunoreactivity of apoB, rLpB and LDL with various mAbs.** The competition curves for the various mAbs described in Figure 11 were analyzed to derive the concentration of apoB protein which results in 50% of maximum (in absence of competitor) antibody binding to the immobilized LDL (ED_{50}). Significant differences in immunoreactivity on addition of phospholipids were seen for conformation specific antibodies 2D8 ($p<0.001$)and 4G3 ($p<0.01$).

Discussion

The direct relationship between low density lipoprotein and cardiovascular disease has led to an intensive investigation of the role of this lipoprotein in cholesterol transport. Since, LDL comprise a complex and heterogeneous pool of lipoprotein particles differing in lipid content, size and density (Chen C G., et al., 1994.and Krauss M.R. and Burke J.D., 1982), the ability to generate homogeneous reconstituted LDL particles having predefined chemical and physical properties provides an important means for identifying specific LDL variables that are important in modulating apoB conformation and function and producing observations which are specific and unambiguous. Reconstitution of LDL has been studied using different methods by various investigators (Lundberg B., et al., 1984 and Kreiger M., 1986, Ginsburg S.G. et al., 1982). Earlier methods of reconstitution involved removing 99% of core cholesteryl esters of LDL by heptane extraction and replacing it with equal amounts of exogeneous cholesterol linoleate in heptane in the presence of potato starch powder (Kreiger M., et al., 1986). Ginsburg et al (1982) prepared reconstituted particles by the interaction of lipid-free sodium deoxycholate solubilized apoB and microemulsions of sonicated cholesteryl oleate and surface stabilized phosphatidylcholine. A variation of the latter method was published by Lundberg et al. in 1984. This method involved the solubilization of apoB from native LDL with sodium deoxycholate; separation of the lipid and detergent complexes by gel filtration chromatography followed by the addition of the isolated apoB to microemulsions of egg yolk phosphatidylcholine. Other approaches that have been used to modulate LDL composition are based on the ability to remodel native LDL by coincubation with VLDL or a triglyceride-phospholipid emulsion in the presence of a cholesteryl ester transfer protein (Aviram et al., 1988a, Aviram et al., 1988b, Kinoshita et al., 1990, McKeone et al., 1993, Galeano et al., 1994). Although these methods provide a way for preparing biologically active particles with altered composition, they do not easily

allow one to systematically study the effects of individual variables in isolation on apoB conformation and function.

A prerequisite for successful reconstitution is the removal of LDL lipids without causing the denaturation of the protein moiety and the production of a water-soluble apoB which is not aggregated. The method of delipidation used in this study involved the solubilization of apoB with NaDOC, followed by chromatographic separation of apoB from LDL lipids. NaDOC is a bile acid with mild effects. It acts as a weakly ionic detergent and functions to dissociate the interactions between lipid and protein molecules effectively. Detergent solubilization yielded mixed micelles of lipids plus detergent and apoB, which were easily separated by size exclusion chromatography. The detergent substituted for the naturally occurring lipid environment of LDL and had the ability to stabilize apoB in the absence of lipids. Under these conditions, apoB is always associated with detergent and therefore not driven to self-aggregate. Lipid analysis of the isolated apoB which showed it to be completely devoid of phospholipids, cholesterol and cholesteryl esters but to have retained 70% of triglycerides from LDL. Since removal of NaDOC had no effect on the solubility/aggregation of apoB, these data suggest that apoB is able to solubilize a significant amount of triglyceride, in the absence of phospholipids, and still remain soluble in aqueous solutions. As such, it is likely that triglyceride association with apoB may be important in maintaining its integrity and preventing self-aggregation. These *in vitro* observations are supported by *in vivo* studies which have shown that the amount of apoB100 that is translocated onto mature VLDL particles depends on the rate of triglyceride biosynthesis (Jonas et al., 1992). Thus, when biosynthesis is increased, most of the apoB forms VLDL particles and when the triglyceride biosynthesis is low apoB is degraded (Jonas et al., 1992). This association of apoB with triglycerides has not been observed by other investigators who have examined LDL delipidation. It is profound that in this study that apoB, an amphipathic protein,

was able to solubilize large amounts of triglycerides, a hydrophobic lipid. A possible explanation is that the triglyceride droplets which form during detergent solubilization contain surface defects or "holes" (Jonas et al., 1992). When specific conditions are met, apoB can stabilize its conformation by attaching to the sites of defects via hydrophobic amino acid residues or a hydrophobic helical face of the protein. This strong affinity of apoB for triglycerides may be important to understanding apoB synthesis and VLDL assembly.

In this investigation the kinetics of phospholipid association with apoB and the effect of this lipid on apoB conformation were analyzed in detail. Unlike other apolipoproteins such as apoA-I, which are unable to associate with POPC, apoB was able to spontaneously and rapidly interact with both DMPC (not shown) and POPC. This strong affinity for phospholipids is expected, since in its native milieu, apoB is intertwined with phospholipids. The addition of phospholipids to triglyceride-associated apoB probably resulted in the rearrangement of the triglyceride vesicles to allow the intercalation of phospholipids resulting in the formation of a small dense particle. This was confirmed by nondenaturing gradient gel electrophoresis which showed that the POPC complexes were of a smaller diameter relative to native LDL. The disruption of the triglyceride lipids on addition of phospholipids and the reassociation of apoB is a rapid process and seems to be intrinsic to the protein. Clearly, apoB has lipid binding domains that can stabilize lipid complexes in the size range of LDL. These results suggest that the manner in which apoB folds is continuously variable, a result consistent with its ability to bind varying amount of lipids *in vivo* (Young S.G., 1989). It is known that apoB forms complexes *in vivo* with 5-20 times its own mass of various lipids and perhaps more importantly can vary its binding capacity as the lipid content is altered during intravascular catabolism of VLDL to the ultimate product LDL (Young S.G., 1989). Therefore, apoB appears capable of a unique conformation adaptability which is dependent and responsive to its lipid environment..

The maximum amount of phospholipid that could be complexed with apoB-TG was found to be between 300 and 400 molecules, which is considerably less than the 600 to 800 molecules/mol protein found in native LDL. This difference in the phospholipidation propensity of apoB-TG may be due to the lack of both cholesterol and cholesteryl esters in the rLpB and may reflect an ability of neutral lipid composition to regulate surface lipid affinity of apoB. The association of phospholipids with apoB-TG was independent of the specific phospholipid transition temperature and occurred at a variety of temperatures ranging from 4°C to 24°C. These results suggest that apoB-TG is sufficiently lipophilic and that the activation energy of lipid-protein interactions is low enough for a rapid association to occur independently of the lipid organization. The lipophilic character of apoB is evident by the fact that this apoprotein has a higher mean residue hydropathy than the exchangeable apoproteins (Phillips M.C., 1992). ApoB is a large hydrophobic molecule with an intrinsic requirement to mask its hydrophobic domains. The addition of POPC to apoB-TG resulted in the intercalation of this lipid into apoB thereby resulting in the concealment of unstable exposed hydrophobic domains.

The unfolding of α-helical segments on apoB complexed to POPC was assessed by analysis of GdnHCl denaturation curves. The GdnHCl induced unfolding of apoB monitored by circular dichroic spectroscopy supported the conclusion that two or more partially stable intermediates are present in the process. ApoB-TG contains a small amount of α-helical structure, that appears to be completely denatured by GdnHCl and exhibit a midpoint of denaturation of almost 3 M GdnHCl. The addition of phospholipids to apoB resulted in a progressive increase in its α-helical content and a significantly reduced propensity to be denatured. This implies that apoB secondary structure and stability is sensitive to the amount of phospholipid bound. Phospholipidated apoB is protected from complete denaturation and undergoes only a partial unfolding of α-helices. The data is consistent with a multi-

phase denaturation, wherein the protein undergoes a stepwise denaturation of unique domains in apoB that are progressively exposed as the molecule loses organized structure. The observation that only 5-15% of the α-helical structure of the POPC-bound apoB can be unfolded by GdnHCL suggests that denaturation does not result in the complete dissociation of apoB from the lipid interface but represents the unfolding of specific exposed domains of apoB on the lipoprotein surface. It would appear that the highly helical conformation of POPC-bound apoB shields the peptide backbone and aromatic residues of the protein from guanidinium ions. This reduction in GdnHCl binding sites may be partially due to apoB being tightly interweaved within the POPC environment. This may result in the protection of aromatic residues, buried deeply within the hydrophobic environment of the α-helical segments of apoB. In contrast, apoB-TG not associated with POPC appears to have more exposed aromatic residues and peptide bonds and yields a less stable apoB that is more easily accessible to GdnHCl and prone to complete denaturation. Since increasing the phospholipid/apoB ratio increases α-helix content, it follows that the α-helical segments of lipidated apoB may be involved in interactions with phospholipids. The stabilizing effect of POPC on apoB suggests that the helical structure of apoB may also be critical to the molecular integrity of the molecule. Both the predicted secondary structure and circular dichroic measurements suggest that apoB may contain up to 40-50% α-helical structure (Steele J.C, 1979). This is consistent with what has been observed with the exchangeable apoproteins; amphipathic α-helices are thought to be important for phospholipid binding and lipoprotein stability (Sparks L. D., Davidson S.W., Katz-Lund S., and Phillips C.M. 1995). In contrast, other studies have suggested that the β-sheet structure of apoB is critical to lipid binding and structural stability of apoB (Segrest J.P., et al., 1994). Yang et al (Yang C.Y., Gu Z.W., Tae W.K., San-Hwan C., Pownall J.H., Sharp M.P., Shyan-Woei l., Wen-Hsiung L., Gotto M.A., and Chan L., 1989) have shown that tryptic apoB fragments that can readily associate with lipid vesicles

originate from regions of apoB primary structure that are predicted to form β-sheets in native LDL (Segrest J.P., et al., 1994).

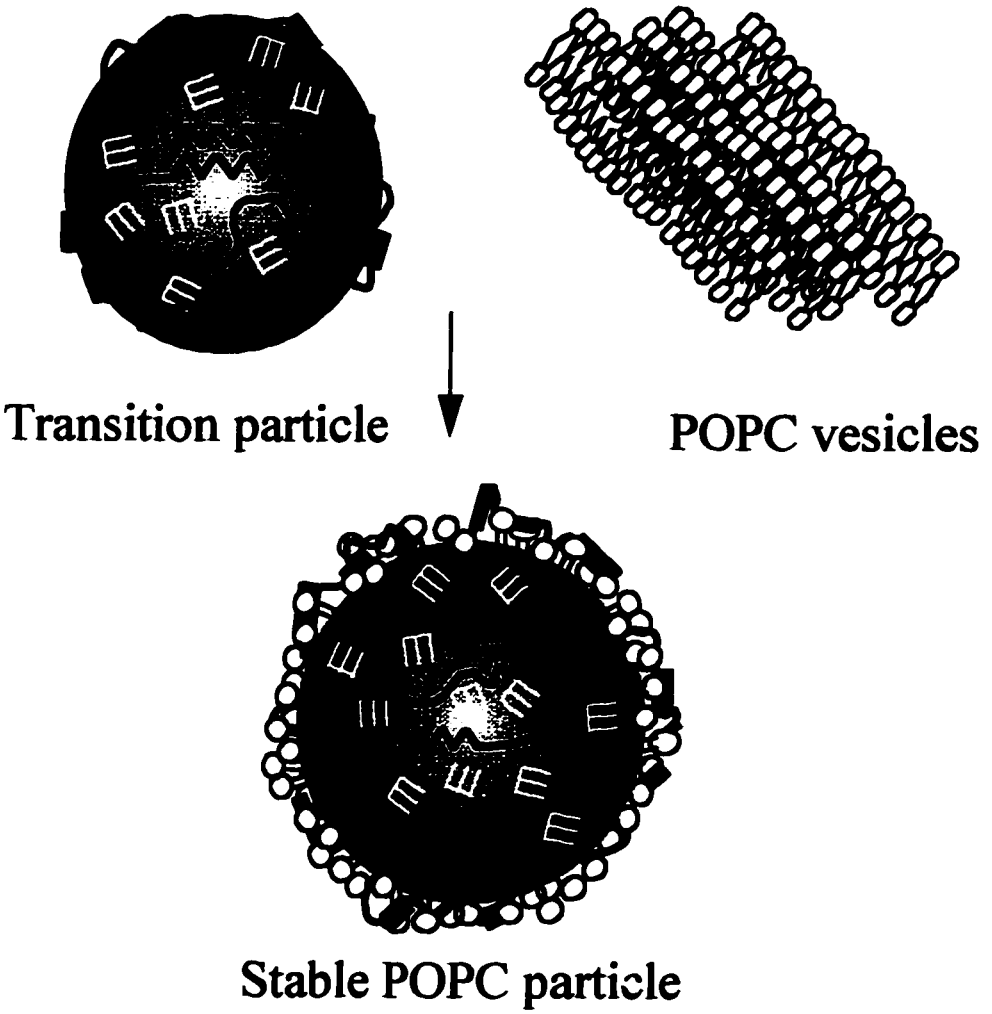

Figure 13: Model depicting apoB association with POPC.

Results from the present investigation suggest that the β-sheet structure in apoB may stabilize the highly hydrophobic domains by governing interactions with neutral lipids, while the α-helices of apoB probably facilitate phospholipid binding and the structural stabilization of the lipoprotein. Taken

together, the data suggest that both the α-helical and β-sheet structure of apoB act in concert to allow for a certain amount of conformational plasticity and yet maintain the lipoprotein structural integrity.

Complexation of apoB-TG with POPC appears to disrupt the interactions between apoB and triglycerides and leads to an increased exposure of unique, negatively charged, domains within apoB. Electrokinetic analysis of apoB-TG and the rLpB showed that the addition of POPC increased net negative charge on apoB by increasing the molecular valence. Our results further show that a relationship exists between apoB surface charge, α-helical content and stability for the different apoB-containing particles (Table 2). An increase in the magnitude of the negative surface potential on the rLpB particles appears to be directly related to an increase in α-helix content and reduced propensity to be denatured. These data suggest that apoB-TG is a metastable "transition particle" that contains an adaptable, but unstable β-structural organization (Figure 13). Intercalation of phospholipids causes the reorganization of the complex and promotes the formation of a more condensed/stabilized lipoprotein particle. The condensation of the particle appears to bring newly exposed negatively charged residues on apoB into closer proximity. This may define unique charged domains in the apoB molecule, domains that have specific metabolic functions.

An unexpected finding in the present study was the ability to detect the expression of a series of apoB epitopes on apoB-TG. The panel of mAbs used in the competitive radioimmunometric assays were chosen on the basis of the distribution within apoB primary structure (Pease et al. 1990) and lipid requirements of their respective epitopes (Marcel et al., 1983). It had been previously reported (Marcel et al. 1983) that, of the five mAbs used in the present study, only 1D1 could recognize delipidated, resolubilized apoB (Cardin et al., 1990). We show here that, in a solid phase radioimmunometric assay, the apoB-TG could compete with native LDL for binding to all five mAbs. The slopes of the competition curves obtained with the apoB-TG were parallel to those obtained with

native LDL which would suggest that the mAbs recognize apoB in the two preparations with similar affinities. On the other hand, the five-fold higher ED_{50}s that were observed with the apoB-TG compared to those seen with native LDL could indicate that the preparations were conformationally heterogeneous and that the epitopes were accessible in only about 20% of the molecules. It is notable that this was the case for all of the mAbs, including 1D1, whose epitope appears to be lipid-independent (Marcel et al. 1983). The ability to detect the 2D8, 3F5, 4G3 and 5E11 epitopes in apoB preparations that had been partially delipidated with cholate and, the lack of expression of these epitopes in apoB that had been delipidated with organic solvents before resolubilization, likely reflects the contribution of the apoB-associated triglyceride in the partially delipidated LDL used in the present study. The triglycerides may allow apoB to adopt and maintain a stable conformation, wherein the various epitopes are exposed in a similar fashion to that of apoB on native LDL.

When the apoB-TG was reconstituted with phospholipid, there was little change in its immunoreactivity with the mAbs. Only the ED_{50} values obtained with mAbs 2D8 and 4G3 were significantly reduced. It had been previously reported that 2D8 reactivity with completely delipidated apoB could be recovered by incorporation of the apoB into DMPC and POPC liposomes whereas, expression of the 3F5, 4G3 and 5E11 epitopes required incorporation of the apoB into cholesteryl ester, phospholipid microemulsions (Marcel et al, 1983). However, in spite of normalization of α-helical content in the apoB-TG following incorporation of POPC, there was little parallel increase in the immunoreactivity. The epitopes therefore appear to be relatively independent of apoB secondary structure but may require the presence of other lipids, such as cholesteryl esters, for maximal expression or accessibility.

The immunoreactivity of many epitopes is variable and, in many cases is dependent on the assay format. In a sandwich assay (Figure 5), antibody 2D8 was found to have a higher

immunoreactivity with apoB-TG relative to native LDL. However, in a competitive radioimmunoassay (Figure 12), 2D8 was found to have a lower immunoreactivity with apoB-TG relative to native LDL. In a competitive radioimmunoassay, the antigen, LDL or apoB-TG is in a soluble form, unbound to microtiter wells. In a sandwich assay, the antigen is bound to microtiter wells through an antibody (1D1). These differences in the state of the antigen is likely responsible for the contradictory immunoreactivity of antibody 2D8 for LDL and apoB-TG.

This study shows that apoB has an inherent ability to strongly interact with triglycerides. When complexed with triglyceride, apoB can be recombined rapidly and spontaneously with phospholipids, to produce well-defined lipoprotein complexes. Immunochemical analysis showed that the rLpB complexes are immunoreactive with a variety of mAbs but express only 2 antigenic determinates in a manner similar to that for native LDL particles. It was shown that the phospholipid/apoB complexes were equivalently immunoreactive as apoB-TG, which indicates the importance of triglycerides in the maintenance of apoB integrity. Circular dichroic spectroscopy and isothermal denaturation studies showed similarities in α-helicity and denaturation propensity of the complexes relative to native LDL. Addition of phospholipids to apoB appears to promote changes in conformation which leads to the formation of more electronegative, stable particle containing a large amount of α-helical structure. Hence, the addition of phospholipids to apoB-TG may result in the transformation of the "transition particle" to a structurally stabilized lipoprotein complex (Figure 13). Further addition of cholesterol and cholesteryl esters molecules to the rLpB complex would be expected to further increase the net negative charge and thermodynamic stability of the lipoprotein particle. Lipid composition induced changes in the charge and conformation of apoB may have major effects on the functional properties of the lipoprotein particles.

Future Studies

The study of the structure and function of native LDL particles has been hampered by their biological heterogeneity and by the technical limitations that have made the isolation of mono-disperse rLpB populations very difficult. To investigate the molecular details of the structure/function relationships involved in LDL metabolism requires the use of well-defined, homogeneous substrates. The work presented in this thesis successfully showed that homogeneous well defined LDL particles containing the lipids to be studied could be prepared and isolated. Once isolated these particles could then be analyzed for protein conformation and integrity using biophysical and immunochemical techniques. The scope for the use of reconstituted particles in understanding LDL structure and functionality is limitless. Future prospects for this study will entail the preparation of reconstituted particles useful for examining the role of cholesteryl esters and cholesterol on the conformation and stability of apoB. These particles can be prepared through a sonication procedure essentially developed by Lundberg B. et al. (1992). The functionality of these particles can be examined through studying their interaction with the LDL receptor and enzymes involved in lipid metabolism. Similar experiments can also be performed using reconsituted particles which differ in size and charge. It has been reported that certain natural truncated apoB variants e.g. apoB75 and apoB90 have a higher affinity for the LDL receptor then does the wild type LDL. Other experiments will entail the preparation of reconstituted particles using truncated apoB species. Similar experiments can also be performed on apoB which is mutated at amino acid residue 3500. This apoB reacts poorly with the LDL receptor and is responsible for the condition known as familial defective apolipoprotein B100.

Together, these studies will provide further insight into the nature of the interactions between LDL apoB, lipids and the LDL receptor.

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