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THE EFFECTS OF LIPOPROTEIN SURFACE CHARGE ON CHOLESTEROL METABOLISM

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ABSTRACT

To examine the role that lipoprotein surface charge plays in cholesterol metabolism *in vivo*, we characterized the effects of an intravenous injection of an uncharged phospholipid (phosphatidylcholine, PC (~8 mg/kg)) or an anionic phospholipid (phosphatidylinositol, PI (~9 mg/kg)) into fasted rabbits. The PI-injection significantly ($P<0.05$) increased the negative surface charge of the lipoproteins. The clearance of tritiated-cholesterol from the PI-injected rabbit-plasma was ~50% greater when compared to controls ($P<0.05$). The PI-injection also prevented the formation of cholesteryl ester. To determine how increased lipoprotein PI content may regulate cholesterol clearance by the liver, cell culture studies using a human hepatoma cell-line were undertaken. *In vitro* enrichment of human plasma or high density lipoproteins with PI caused a ~2 fold stimulation in cholesterol cellular uptake, relative to controls. PI-enrichment did not affect cellular cholesterol-uptake from low density lipoproteins. These results suggest that lipoprotein PI levels may affect intravascular cholesterol transport.

ABSTRACT

The electrical charge properties of plasma lipoproteins can directly affect cholesterol metabolism. These charge properties are governed by the lipid and protein compositions of the lipoprotein particles. To examine the role that lipoprotein charge plays in cholesterol metabolism *in vivo*, we characterized the effects of an intravenous injection of an uncharged phospholipid (phosphatidylcholine, PC (~8 mg/kg)) or an anionic phospholipid (phosphatidylinositol, PI (~9 mg/kg)) into fasted rabbits. PC-injection had a negligible effect on lipoprotein charge. In contrast, PI-injection caused a significant increase in the net negative surface charge of all lipoproteins, followed by a gradual return to normal by 24 hours. Lipoprotein compositional analysis showed no conclusive change in cholesterol (FC), cholesteryl ester (CE), or triglyceride mass levels. Radioactive clearance of co-injected tritiated-FC showed a ~50% greater initial clearance rate ($P < 0.001$) of the tracer from the PI-injected rabbit plasma as compared to PC-injected controls. Data suggest that PI stimulated the clearance of radioactivity from the plasma and prevented the formation of CE, as the rate of cholesterol esterification by lecithin:cholesterol acyltransferase (LCAT) was inhibited in the PI-injected rabbit. The addition of 1 mg/ml of PI-vesicles to either rabbit or human plasma inhibited the esterification rate of cholesterol *in vitro*.

To determine how increased lipoprotein PI content may regulate cholesterol metabolism in the liver, cell culture studies using a human hepatoma cell line, HepG2, were undertaken. Incubations of the cells with plasma or HDL enriched with PI-vesicles (0.2 mg/ml-plasma or 0.2 mg/mg-lipoprotein) showed a ~2-fold stimulation in FC

cellular uptake, relative to incubations with plasma or HDL enriched with PC-vesicles. In contrast, PI-enrichment of LDL had no effect on FC-uptake by HepG2 cells, relative to PC-enrichment. Treatment of the cells with the inhibitors, chelerythrine chloride (a protein kinase C inhibitor), or propranolol (a phosphatidic acid phosphohydrolase inhibitor), inhibited FC-uptake in HepG2 cells from HDL, which suggested that these cell-signaling pathways may play a role in the PI-dependent FC-uptake pathway. These results suggest that lipoprotein PI levels, and surface charge may affect cholesterol transport in the plasma.

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ABBREVIATIONS

ABCA-I	Adenosine triphosphate binding cassette protein
ACAT	acyl-coenzyme A: cholesterol acyltransferase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CHO	Chinese hamster ovary
FBS	Fetal bovine serum
FC	Free cholesterol
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
LCAT	Lecithin cholesterol acyl transferase
LDL	Low density lipoprotein
LPL	Lipoprotein Lipase
MDR1	Multi-drug resistance 1 protein
mdr2	Multidrug Resistant 2 Protein (mouse)
MDR3	Multi Drug resistant 3 Protein (human)
PAPH	Phosphatidic acid phosphohydrolase
PBS	Phosphate buffer saline
PC	Phosphtidylcholine

PI	Phosphatidylinositol
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PMA	phorbol 12-myristate 13-acetate
PS	Phosphatidylserine
Spgp	Sister of p-glycoprotein
SR-BI	Scavenger receptor-class B type I
TG	Triglyceride
TLC	Thin layer chromatography
VLDL	Very Low density lipoprotein

INTRODUCTION

Atherosclerosis

Atherosclerosis causes more human deaths than any other disease in the developed world (1). Atherosclerosis is a progressive disease that involves the deposition of cholesterol and other lipids in the intima of the arterial wall, resulting in a narrowing of these vessels and gradual restriction of blood flow (2; 3). As the disease develops, secondary clinical events occur as plaque accumulates causing sufficient stenosis to impede blood supply. The formation of blood clots at the damaged area, termed thrombosis, can occur and lead to complete vessel obstruction, the usual cause of acute myocardial infarction. The disease has been investigated intensely over the past several decades and several mechanisms leading to its development have been elucidated (4).

The basic pathogenic understanding of atherosclerosis involves the response to injury hypothesis. This theory suggests that subtle forms of endothelial dysfunction induce the expression of certain chemotactic agents that promote the adhesion of blood monocytes to the endothelium (1). The intercellular tight junction complex of the endothelium is a semi-permeable barrier between blood and tissue, which permits the diffusion of small particles (i.e., lipoproteins). However, following monocyte adhesion to the endothelial cells, even monocytes migrate into the sub-endothelial space and subsequently differentiate into macrophages (1). Initially, macrophages remove cytotoxic and pro-inflammatory oxidized low density lipoproteins and apoptotic cells from the damaged area and serve a protective function (4; 5). Macrophages also take up unmodified low density lipoproteins (LDL) through an endocytic process mediated by the LDL receptor (4; 5). In this process, the cholesterol carried by LDL acts as a negative

feedback mechanism to down-regulate the expression of the LDL receptor, which controls LDL uptake (8). Brown and Goldstein discovered that modified forms of LDL, acetylated LDL and oxidatively damaged LDL, are taken up by a process that does not involve the LDL receptor (10). It was later understood that macrophages engulf modified LDL at an uncontrolled rate via scavenger receptors (11-13). These modified LDL act as a positive feedback signal to upregulate expression of scavenger receptors, which in turn amplify the cholesterol engulfment process (11). This uncontrolled recruitment of cholesterol results in the transformation of macrophages into the cholesterol laden "foam cells", a hallmark of atherosclerosis (1; 2; 11; 14; 15).

Epidemiological studies have shown a strong relationship between plasma cholesterol levels and the risk of developing atherosclerosis (16-18). The correlation between serum cholesterol and the prevalence of atherosclerosis has been examined in detail. The serum LDL-cholesterol levels have proven to be a more powerful predictor for the development of atherosclerosis than serum-cholesterol levels (19). The levels of LDL-cholesterol in the blood stream are thought to correlate with the levels of modified or oxidized LDL in the body (20). The majority of the oxidized LDL is found in the intima, suggesting that it is the prime location for the oxidative modification of LDL. This oxidized LDL rich environment will subsequently lead to increased cholesterol rich foam cell formation (20). There are numerous environmental and genetic risk factors involved in the development of atherosclerosis. However, unlike other risk factors, elevated serum cholesterol levels can drive the development of atherosclerosis independently (20).

General Cholesterol Metabolism

Cholesterol plays a very important role in normal cell function and is the precursor of steroid hormones and bile acids. Cell membrane fluidity and integrity is also dependent on the presence of cholesterol. However, excess cholesterol can be toxic to cell function and can disrupt the plasma membrane (21). It is also known that high levels of cholesterol play a role in the formation of gallstones and as previously mentioned atherosclerotic plaques (22; 23). Therefore, cholesterol metabolism must be carefully controlled. A typical western diet contains approximately one to two grams of cholesterol per day. A fraction (60-80%) of the dietary cholesterol is absorbed by the body (24) while the remaining 20-40% is excreted in the faeces. After food consumption, cholesterol from the diet is emulsified to a micellular state by fatty acids from the diet and bile acids that are secreted from the gall bladder (24). Cholesterol is taken up by the intestinal cells at the brush border surface. It was initially believed that cholesterol absorption occurred exclusively via monomolecular simple passive diffusion, where the micelle itself did not penetrate the cell membrane (24). The mechanism by which absorption occurs has been further investigated and numerous cholesterol receptors and transporters expressed in the intestines have been proposed to assist in cholesterol absorption. Krieger and colleagues believe that scavenger receptor class B type I (SR-BI), a receptor found in the intestinal brush border, may mediate an interaction with the cholesterol-rich micelle (25). The adenosine triphosphate (ATP) binding cassette (ABC) transporter, ABCA-I, has been proposed to play a role in intestinal cholesterol absorption (26; 27). Studies have shown another member of the ABC family, a p-glycoprotein encoded by the multi-drug-resistant 1 (MDR1) gene, is involved in FC-uptake and

cellular transport in the intestinal brush border. In these studies, MDR1 was transfected into intestinal cells and was shown to enhance cellular cholesterol uptake from lipid micelles (28). Cholesterol is absorbed by the intestines from the gut and secreted in chylomicrons into the lymphatic and blood systems (24). The human body can synthesize cholesterol and therefore any cholesterol absorbed from the diet is considered excess. All but a few eukaryotic cells have the ability to synthesize cholesterol. Cholesterol synthesis revolves around the function of the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, which regulates the synthesis of cholesterol (29). In the absence of lipoproteins, intracellular HMG-CoA reductase uses acetyl-CoA to trigger a multiple step anabolic pathway to synthesize cholesterol molecules. By controlling the expression of the enzymes in this pathway, the cell is able to maintain cholesterol homeostasis (29).

Excess amounts of cholesterol in cell membranes cause dysfunction and therefore must be transported for storage in cellular lipid droplets (30-32). The storage form of cholesterol is the hydrophobic molecule, cholesteryl ester (CE), which possesses an acyl chain, instead of the 3' hydroxy group. In the cell, the enzyme acyl-coenzyme A: cholesterol acyltransferase (ACAT) regulates intracellular esterification and the FC to CE conversion (33). ACAT is found in the endoplasmic reticulum of the cell where CE is stored. CE is packed in lipid droplets allowing for high levels of cholesterol to be stored in a relatively small area (29; 34). In the hepatocyte, CE is sequestered, but remains accessible to the secretion process of the cell. When required, a CE-hydrolase can hydrolyse the acyl chain to form FC to be used for cellular function or transport (29; 35).

The FC form is transported in intracellular lipid rafts or vesicles. These lipid rafts contain proteins that target the raft to the correct area in the cell (36-38).

Lipoproteins

Cholesterol, due to its hydrophobic properties, cannot exist in an aqueous environment, such as plasma, without the assistance of a carrier lipid/protein complex. Plasma lipoproteins perform the function of transporting hydrophobic lipids, such as cholesterol, through the aqueous blood and possess the structure of a micro-emulsion in solution (39). Lipoproteins contain a polar-lipid monolayer composed of phospholipids and FC, which shield the neutral core lipids (triglyceride (TG) and CE) from the surrounding environment (39). Unique proteins, termed apoproteins (apo), interact with the lipid micro-emulsion (39; 40). Apoproteins are amphipathic and contain domains that contact both the hydrophobic lipid region and the hydrophilic aqueous environment. The apoproteins provide structural integrity to the particle, act as ligands to bind to cell receptors, activate enzymes, and facilitate lipoprotein secretion. Lipoproteins are heterogeneous molecules but can be categorized into different classes based on their size and density, which are dependent on the protein to lipid ratios (41).

Lipoprotein Surface Charge

Lipoproteins are unique transport molecules and display compositions that are highly variable and heterogeneous (42). Unlike cell membrane composition, which can be actively maintained by the cell, the plasma lipoprotein composition is dependent on the surrounding environment. The surface layer composition of lipoproteins is an important factor in lipid metabolism, as the interfacial enzymes and receptors must first interact or bind to this layer (43-45). A compositional dependent property that directly

affects these interfacial interactions is the surface charge characteristic of the lipoprotein particle (46; 47).

Electrophoresis has been traditionally used to classify the different classes of lipoproteins and has played an important role in characterisation of dyslipidemias through the Fredrickson, Levy and Lees' classification scheme (48). Electrophoretic mobility has been defined by the nomenclature alpha, beta, and pre-beta to describe high density lipoprotein (HDL), LDL and very low density lipoproteins (VLDL), respectively (49) (Figure 1). These terms were originally used to reflect the equivalent migration patterns of standard globulin serum proteins. In 1993, Sparks and Phillips developed a technique using agarose gel electrophoresis to measure lipoprotein charge quantitatively and concluded that variations in mobility reflect differences in particle charge (50). The effects of lipoprotein compositional changes on surface charge could be evaluated by tracking an alteration in electrophoretic mobility. Subsequent studies have shown that lipoprotein charge affects lipid metabolism *in vitro* by regulating the interactions with various lipid transfer proteins. Lagrost and colleagues have reported that the lipoprotein surface charge properties represent a major factor that determines the lipid transfer activity for the cholesterol ester transfer protein (CETP) and the phospholipid transfer protein (51; 52). The effects of lipoprotein surface charge on the activity of other proteins and enzymes have not been fully evaluated.

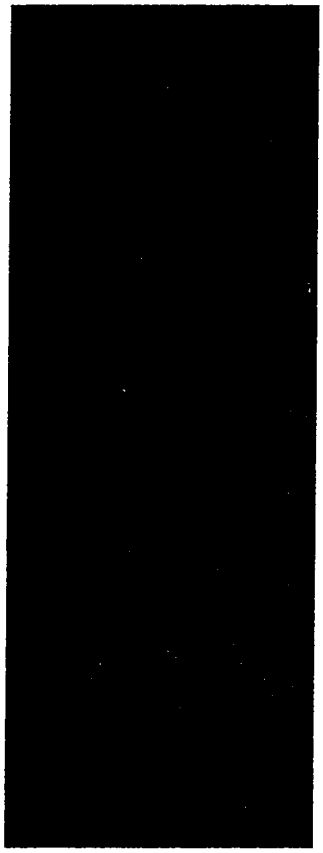
The protein composition of plasma lipoproteins can directly affect surface charge properties (50; 53) (Figure 1). The conformation of the protein, which is affected by the lipid composition, will alter the exposure of certain regions of a protein and thereby alter the particle charge (53; 54).

Figure 1. **Lipoprotein Surface Charge.** Electrophoresis has been used to classify the different classes of lipoproteins and has played an important role in characterization of dyslipiedimias through the Fredrickson, Levy and Lees' classification scheme. (Left) Lipoproteins are separated on the basis of surface charge on a 0.5% agarose gel. HDL has been described as having an α-electrophoretic mobility while LDL has a β-electrophoretic mobility. (Right) One of the major factors able to alter lipoprotein surface charge is the composition and the conformation of the protein. The phospholipid composition is another factor that alters the lipoprotein charge and is dependent on the head groups of the phospholipids (phosphatidylinositol (PI), phosphatidylserine (PS) and free fatty acids (FFA)) that make up the surface layer.

Surface potential

HDL α
-11 mV

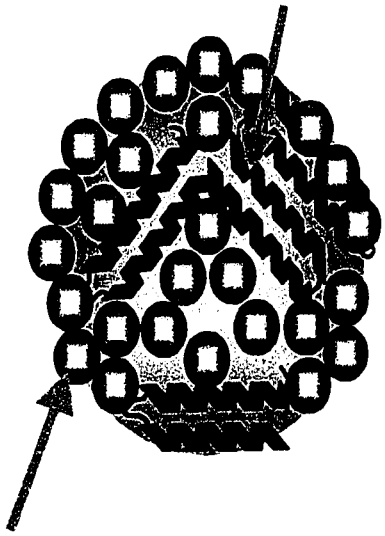
LDL β
-6 mV



Agarose Electrophoresis

Lipoprotein

90% of charge is from apoprotein



10% due to anionic lipids = FFA, PI, PS

The phospholipid composition of a lipoprotein can also directly alter lipoprotein surface charge. The lipoprotein surface charge is sensitive to the head group structure of the phospholipids that make up the surface layer (50; 53). Fatty acids released during the lipolysis of TG during a postprandial response are also thought to increase the net negative charge of the lipoproteins (55) and it has been suggested that fatty acids may stimulate the transport of cholesterol to the liver (56).

Phosphatidylinositol (PI) is the most common anionic phospholipid in the plasma, occupying 5% of the total phospholipid content in HDL. Changes in the PI-lipoprotein composition have been shown to have significant effects on the surface charge of lipoprotein (55). Intracellular PI metabolism also plays an important role in many cell-signalling pathways (57). Cells maintain the balance of anionic phospholipids on the inner-leaflet of the membrane using transporters termed flippases and floppases. However, the plasma levels of PI are not very well controlled. PI is produced by the intestinal cells and is an important structural component for the intestinal secretion of the chylomicrons. Hamsters fed a diet deficient in inositol, a metabolic precursor of PI, accumulate lipids in the intestines due to an inability to secrete chylomicrons (58; 59). Large amounts of PI are distributed to the serum via chylomicrons, resulting in a mass movement of PI to the HDL pool, as the chylomicrons are metabolised in rabbits (60). Therefore, this suggests that HDL may become enriched in PI during a postprandial response when plasma chylomicron levels are high.

Lipoprotein Metabolism

Chylomicrons, found in the blood predominantly in a postprandial state, is the term given to the lipoprotein with the lowest density (41). As mentioned previously, chylomicrons are formed in the intestines. It is here that the dietary lipids such as TG and cholesterol are combined with apoB-48 to form a mature particle (62). The function of chylomicrons is to transfer fats from the diet to the blood stream for delivery to the tissue. An enzyme found in the plasma, lipoprotein lipase (LPL), hydrolyses TG to release free fatty acids and glycerol. LPL requires the interaction with a specific apoprotein, apoCII, for maximal activation (63). LPL hydrolyses TG from chylomicron to form smaller chylomicron remnants, which are rapidly cleared from the plasma (64; 65). The lipids that are taken up by the liver can be recycled as plasma lipoproteins. One class of hepatic derived lipoprotein is called very low density lipoproteins (VLDL). The main protein component of VLDL is apoB100 (66). Unlike apoB48, apoB100 facilitates the binding and subsequent uptake by the LDL-receptor (62). VLDL is hydrolysed by LPL and hepatic lipase (HL) to form smaller classes of lipoproteins such as the intermediate density lipoproteins (IDL), and LDL (67). In addition to apoB100, the LDL-receptor also binds lipoproteins containing apoE (68). The LDL receptor mediates a unique uptake pathway, which is termed receptor-mediated endocytosis (67; 69; 70). The ligand and receptor are internalized in a clathrin-coated vesicle that becomes uncoated and acidified, and results in the formation of an endosome (70). The lipoprotein particle is delivered to the lysosomal compartment, while the receptor travels back to the cell surface through a recycling endosome. The lysosomal contents are then degraded resulting in apoB-degradation and the liberation of FC through hydrolysis of CE. The excess FC can be

reconverted to CE through the enzymatic action of ACAT and stored in lipid droplets (70). In hepatocytes, this cholesterol can be used for the synthesis of bile acids or can be re-secreted into the plasma in a VLDL molecule (70).

Table I. Physical Properties of Human Lipoproteins

Class	*Density (g/ml)*	*Diameter (nm)*
Chylomicron	< 0.93	75 – 1200
VLDL	0.93 - 1.006	30 – 80
IDL	1.006 - 1.019	25 – 35
LDL	1.019 – 1.063	18 – 25
HDL_2	1.063 – 1.125	9 – 12
HDL_3	1.125 – 1.210	5 – 9

Adapted from reference (62)

The smallest class of lipoproteins, HDL, are also divided into classes based on size and density, with HDL_1 being the largest, followed by HDL_2, HDL_3 and very high density lipoproteins (71). The main protein component of HDL, apoA-I, is secreted by the liver and intestine and rapidly forms nascent particles composed mainly of phospholipid, FC and apolipoproteins (62; 72). ApoA-I is secreted into the blood attached to chylomicrons (73) and VLDL, where it dissociates following TG hydrolysis to form HDL (74). ApoA-I is a 243-residue protein that contains amphipathic alpha helices that are thought to be responsible for binding to lipids (75). A great deal of research has been performed to determine how apoA-I interacts with HDL and how it functions to stabilize the size and structure of the particle. Evidence suggests that the stability of apoA-I on HDL may be dependent on the types of phospholipids making up the surface lipids (53; 76-78).

High cholesterol levels that are associated with HDL are strongly correlated with a decreased risk in developing atherosclerosis (79; 80). This anti-atherogenic property of

HDL-cholesterol is associated with many of the components that make up the HDL particle. HDL has antioxidant properties, which are thought to contribute to the inverse relationship to atherosclerosis development (81). It also possesses anti-inflammatory properties that play a role in its anti-atherogenic propensity (82). However, the major function of HDL is its involvement in the reverse cholesterol transport pathway (86; 87).

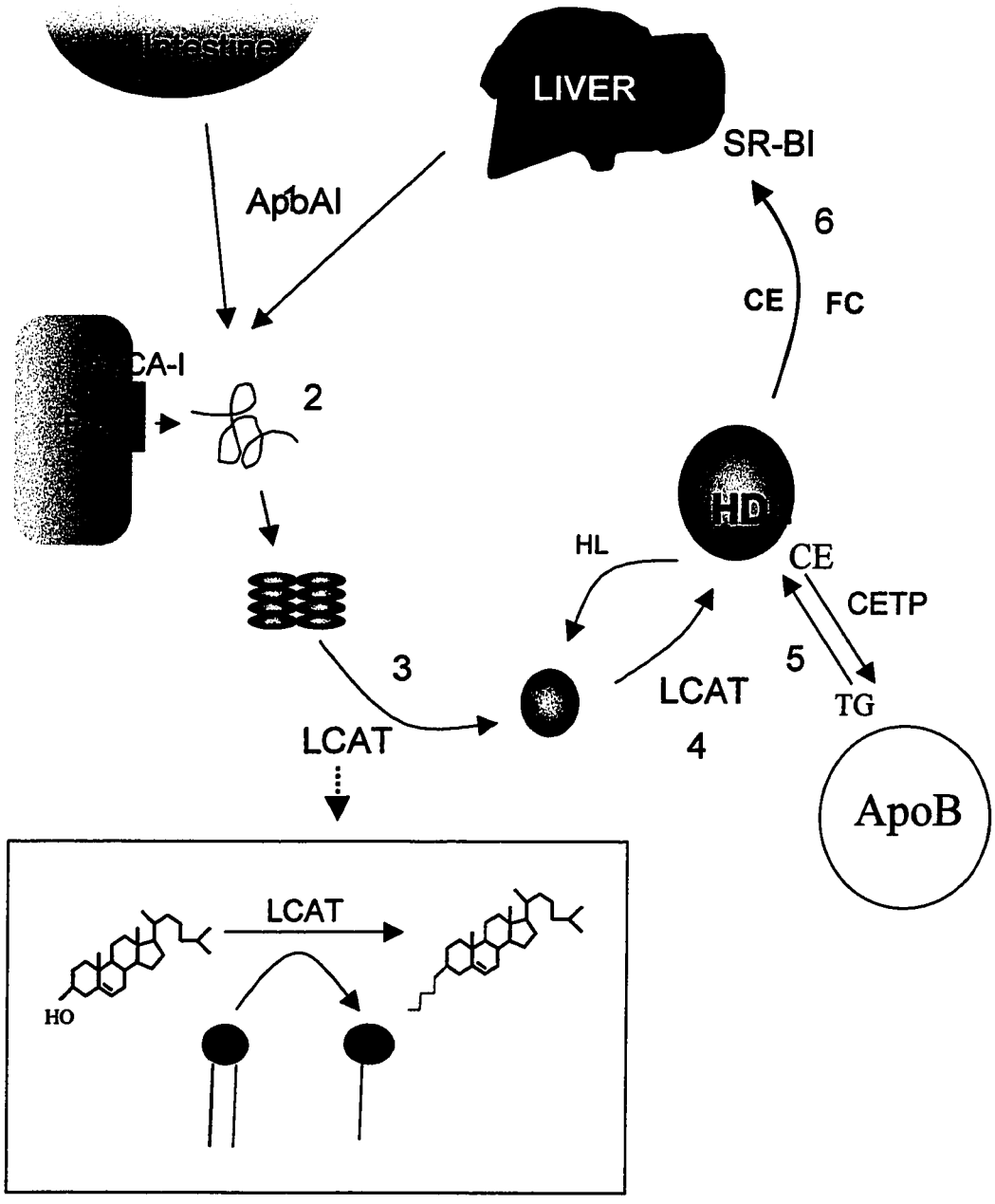
Reverse Cholesterol Transport

Reverse cholesterol transport is the movement of cholesterol from peripheral cells through the plasma compartment to the liver (Figure 2) (86; 88). This transport pathway involves four main steps that include cholesterol efflux from peripheral tissue, cholesterol transport through the blood system, hepatic cholesterol uptake and biliary cholesterol secretion (86; 88). Each of these steps involves several transporters, lipid carriers, enzymes, and receptors to assist in the delivery of the cholesterol molecule.

Cholesterol Efflux

The initial stage of HDL and reverse cholesterol transport is the absorption of FC from peripheral cells into the surface layer of the lipoproteins in the blood (72). Nascent HDL is small and contains no CE and minimal amounts of phospholipids and FC. Maturation of HDL particles requires the adsorption of phospholipids, FC, and sphingomyelin from the cells of the peripheral tissue. The exact mechanism of the lipid efflux is still unclear, but is thought to involve passive diffusion. Cholesterol efflux through passive diffusion is dependent on the principle that FC can passively move through an aqueous phase from the plasma membrane to plasma HDL. This mechanism requires lipoproteins to be in close proximity to the plasma membrane to facilitate the

Figure 2. **Reverse Cholesterol Transport Pathway** (1) ApoA-I, the major protein associated with HDL, is secreted into the plasma from the liver and intestines. (2) Excess free cholesterol (FC) diffuses across the cell membranes of peripheral tissue to lipid poor ApoA-I, a process dependent on the action of ATP binding cassette (ABCA-I) transporter. (3) Lecithin cholesterol acyl transferase (LCAT) converts FC to cholesteryl ester (CE) through the transfer of an acyl chain from phospholipids to the 3`C. (3, 4) The newly formed HDL is ultimately converted to the larger spherical HDL. (5) CE can then be transferred by cholesteryl ester transfer protein (CETP) from HDL to apoB containing lipoproteins in exchange for triglycerides (TG). (4) Nascent HDL is regenerated via hepatic lipase (HL) and the cycle continues. (6) The HDL interacts with the liver directly through the scavenger receptor BI (SR-BI), which is involved in the selective uptake of FC and CE by the hepatocyte.


Intestine
LIVER
SR-BI
ApbAI
6
CE
FC
CA-I
2
HD
HL
CE
CETP
5
TG
3
LCAT
LCAT
4
ApoB
LCAT
HO

movement of cholesterol through the aqueous phase and down the concentration gradient (89-92).

A new mechanism for cellular FC-efflux has emerged in the last few years from investigations into the pathogenesis of a unique autosomal recessively inherited disorder called Tangier disease (93; 94). Tangier disease was named after the island (Tangier, Virginia) where individuals with the disease were first characterized. These individuals had a unique serum lipid phenotype and exhibited very low levels of HDL (95; 96). Recent findings in 1999 have shown that a dysfunction of the plasma membrane transporter, ABCA-I, found in the ATP binding cassette transporter family was linked to this disease (98). ABCA-I is a large 254 kDa transmembrane protein, containing two transmembrane domains with each one containing six hydrophobic membrane spanners (97). The transporter also possesses two nucleotide-binding regions on the cytosolic region. These regions regulate the function of the transporter through binding of ATP (97). It is now known that this transporter plays an important role in efflux of cellular cholesterol to the apoA-I molecule (97). Cholesterol efflux has been reported to be sensitive to the cellular levels of cholesterol and protein kinase C (PKC) and protein kinase A (PKA) -dependent signalling pathways (99; 100; 199). These pathways presumably work in concert to regulate the cholesterol efflux route and reverse cholesterol transport. ApoA-I may directly interact with ABCA-I, which is known to form a channel on the plasma membrane where lipids can be transported (97). Alternatively, the transporter can act as a flippase and transport lipids to the acceptor lipoprotein (103). ABCA-I plays an integral part in the initial stages of the reverse cholesterol transport pathway, as it transfers cholesterol out of the cell to an accepting

nascent lipid poor apoA-I molecule (104). Recent studies report that the over-expression of ABCA-I in mice leads to increased levels of apoA-I in the plasma and an increased biliary excretion of phospholipids and cholesterol, outlining the role of the transporter in the reverse cholesterol transport pathway (105).

Cholesterol Vascular Transport

Once on the surface of native HDL, cholesterol becomes the substrate for the enzyme, lecithin-cholesterol acyltransferase (LCAT). LCAT transfers an unsaturated fatty acid chain from the sn-2 position of lecithin to the 3-hydroxy group of cholesterol and thereby generates CE and lysolecithin (106; 107). LCAT is a 416-residue serine esterase that is secreted into the plasma by the liver (108). LCAT actively hydrolyses phospholipids. In the presence of FC, LCAT transfer the newly formed acyl group to form CE (108). Similar to lipases, LCAT is an interfacial enzyme, and requires binding to the lipoprotein surface prior to performing the catalytic function (76). HDL is the preferred substrate for LCAT, due to the presence of apoA-I, which activates the enzyme (109). Data suggest that it is the direct interaction of charged residues on both the apoA-I and LCAT proteins that cause the activation (110). Other studies suggest that apoA-I may activate LCAT by increasing its dissociation rate, therefore allowing it to relocate to another area on HDL (111). Studies by Sparks *et al* have shown that both the apoA-I conformation and the surface composition of HDL influence the activity of the enzyme (45; 76).

The enzymatic action of LCAT causes the nascent HDL particle to undergo a structural change, where FC on the surface becomes partitioned to the core as a CE molecule (72). This conversion of FC to CE causes the molecule to become more

hydrophobic and results in the internalization of cholesterol to the inner core of the lipoprotein. The movement of CE to the inner region of HDL results in expansion of the core and the formation of a more spherical lipoprotein structure. The theory, originally developed by Glomset, suggested that the esterification of cholesterol causes the movement of CE to the inner core of HDL allowing space for another molecule of FC to be absorbed from the cell surface (72; 112; 113). These spherical particles have been shown to be much more stable and possess lower fractional catabolic rates than nascent particles in the body (114). Patients with inactive LCAT, due to a mutation in the protein, have very little HDL, low levels of HDL-cholesterol and accumulate cholesterol in peripheral tissue. However, it remains unclear what role LCAT has in the progression of atherosclerosis (115-117). Studies have reported that LCAT-deficient mice have reduced diet-induced aortic atherosclerosis compared to normal mice (118; 119). Despite low plasma HDL levels in patients deficient in LCAT, the risk of atherosclerosis did not increase (120). Likewise, LCAT-over-expressing mice have elevated levels of HDL but exhibit either no protection against atherosclerosis or have enhanced development of aortic lesions (121). Individuals with decreased activity of LCAT also have lower levels of cholesterol in LDL and VLDL (122), suggesting a potential advantage to the reduction of LCAT activity. LCAT therefore appears to regulate the storage of CE by modulating the transport of cholesterol from the tissues to the LDL storage pool (123). The accumulation of CE in LDL increases the risk of developing atherosclerosis. It seems that while LCAT is required to maintain a mature HDL pool, high activity does not necessarily enhance reverse cholesterol transport.

The cholesterol in the mature HDL particles can have several fates. Although FC and CE in HDL are similar in structure, they are metabolized in very different ways. The hydrophobic sterol, CE can be transferred to other lipoprotein particles through the action of the transfer protein, CETP. CETP is a hydrophobic glycoprotein that is secreted by the liver, and is found predominantly bound to HDL in the plasma (124). This transfer activity was first discovered when Nichols and Smith reported that there was a reciprocal transfer of cholesterol and TG between lipoproteins, during incubation with human plasma (125). The action of CETP can be described as a bridge to allow the passage of hydrophobic lipids down a concentration gradient between lipoproteins (126). The regulation of the initial binding of CETP to the lipoprotein is thought to be electrostatic in nature, and the presence of a negative surface charge is believed to play a role in this process (126). Previous unpublished data showed that the incubation of PI with plasma *in vitro* caused a transport of cholesterol from LDL into HDL, suggesting an alteration in CETP activity (Camlioglu and Sparks, unpublished observation). The interaction of the positively charged residues of CETP with the negative charge of the lipoprotein stabilises the CETP-lipoprotein complex (126). Some studies also show that CETP may play a role in the delivery of cholesterol to the tissues through unique cell surface interactions (127-129). The role of CETP in the development of atherosclerosis is not well understood, and it is thought to have both atherogenic (130; 131) and anti-atherogenic properties (132; 133). The formation of CE and its subsequent transfer to VLDL and LDL by CETP, is thought to be a part of the reverse cholesterol transport pathway and therefore anti-atherogenic (134; 135). After being transferred to VLDL and LDL, cholesterol is delivered to the liver by the LDL-receptor, as previously described. Data that are more

recent suggest that HDL can directly interact through specific HDL-receptors expressed on the liver (136).

Cholesterol Uptake

Over the past ten years, several potential HDL receptors have been proposed. It was shown that a receptor complex, called megalin/cubulin, can mediate holo-particle uptake of HDL and was speculated to be involved in renal re-absorption and in the maternal-fetal transport of cholesterol (137; 138). However, cubulin and megalin have not been shown to play a role in HDL hepatic uptake. Another candidate HDL receptor, the HDL binding protein, HB2, has been shown to increase HDL binding when over-expressed in cells (139). The physiological significance of HB2 in HDL metabolism has not been identified. Scavenger receptor, CD36, has been identified to bind HDL (140) although it is not thought to play a direct role in cholesterol metabolism (141). It is now believed that cholesterol uptake by the liver occurs through the interactions of HDL with the scavenger receptor class B type I (SR-BI), originally identified in mice (101).

SR-BI or the human homologue, CLA1 (142), is expressed in the liver, although it is found in high concentration on steroidal tissue such as the adrenal glands, ovaries, intestines and testis (143-145). SR-BI has been shown to bind anionic phospholipid-vesicles, native LDL and HDL, and apoproteins associated with HDL (146). Unlike the LDL-receptor, which mediates cellular endocytosis and lysosomal degradation of the entire lipoprotein, SR-BI mediates cholesterol uptake via a mechanism called selective uptake (147; 148). Selective uptake of cholesterol is a mechanism where cholesterol, FC and CE, are taken up from lipoproteins without a concomitant uptake or degradation of the lipoprotein particle (149; 150). The mechanism by which SR-BI mediates the uptake

of cholesterol is still under study. One opinion is that HDL interacts with SR-BI allowing FC to diffuse to the plasma membrane and CE to funnel into the cell via a hydrophobic pore formed by the receptor (151). An alternative mechanism suggests that SR-BI allows for holo-particle uptake by the hepatocytes, where the cell retains the lipids and the cholesterol-depleted particle is re-secreted to the circulation via an endocytic-recycling compartment (152).

Some landmark atherosclerotic studies enhanced SR-BI expression using adenovirus expression in SR-BI knockout mice. SR-BI over-expressing mice had lower levels of serum cholesterol, HDL, and HDL-cholesterol, and a decreased progression of atherosclerosis (153, 154). These studies suggest that SR-BI plays an important role in the reverse cholesterol transport pathway in mice.

Cholesterol Excretion

The liver is the major route for elimination of cholesterol, as it expresses both receptors for LDL and HDL and has the ability to filter and secrete waste into the faeces via the gall bladder (155). Hepatic cholesterol can be eliminated directly as FC or (after synthesis) as bile acids (156). The liver secretes lipids (cholesterol, phospholipids), bile acids, water and electrolytes to form a complex fluid called bile (157-159). Bile is targeted to the bile ducts where it obtains bicarbonate-rich water from the epithelial cells of the duct (158; 159). The bile is then retained in the gall bladder for storage where it is concentrated through the active removal of water (160). Bile functions as a medium for waste products and toxins that have been absorbed by the liver (161). The bile also aids in the digestion of fats and vitamins in the small intestines (162; 163). The presence of chyme in the duodenum triggers the release of bile from the gall bladder into the small

intestines. Once mixed with the chyme, the bile dissolves the fats and lipids allowing digestion to occur (162; 163). Materials in the bile that are not reabsorbed by the intestine are excreted in the faeces.

The main functional components in the bile are the bile acids, which dissolve the dietary lipid and facilitate absorption (164). Once in the intestines, efficient Na^+/bile acid co-transporters on the enterocytes reabsorb the majority (95%) of the bile acids secreted in the bile (165). The bile acids then enter the circulation through the portal vein where they are absorbed by the hepatocytes. Approximately 5% of the bile acids are synthesized from cholesterol by the liver (156). The bile acid synthesis pathway is regulated by the rate-limiting enzyme 7-hydroxylase or CYP7A1 (166). The expression of this enzyme is controlled on a transcriptional level by several nuclear transcription factors. The activation of CYP7A1 is dependent on the relative ratios of cholesterol and bile acids in the liver (166). The anabolism of cholesterol to bile acids requires energy and it is thought that bile acids do not represent the major cholesterol elimination pathway in the body (156). The hepatic FC secreted in the bile that is derived from plasma lipoproteins, probably represents a more significant route for sterol elimination by the reverse cholesterol pathway (167).

Hepatocytes express polarity in order to meet the requirements of the different environments on each apical and basolateral membrane domains (Figure 3) (168). The types of hepatic proteins and receptors are unique to each membrane and require the use of the trans-Golgi network to direct cellular transport (168). The basolateral (sinusoidal) membrane of hepatocytes faces the circulation and therefore expresses high levels of lipoprotein receptors, such as SR-BI, LDL-receptor and the bile acid transporter (156).

The collective apical or canalicular membrane of hepatocytes forms a channel termed the bile canaliculus (156). The canalicular membrane regulates the transport of cholesterol, bile acid and phospholipid into the bile (156). The machinery employed to regulate hepatocyte cholesterol metabolism is very intricate and has been studied for many years. It is well known now that cholesterol from LDL, HDL and de novo synthesis are utilised in different ways by the hepatocyte (156). Once presented at the inner surface of the canalicular membrane, it is thought that cholesterol is transported into the bile via a passive diffusion mechanism (168). Transport of bile acids and phospholipids across the canalicular membrane require the action of ATP-dependent transporters. Hepatocytes express high levels of a p-glycoprotein transporter, encoded by the multi-drug-resistant 3 (MDR3) gene in humans or mdr2 gene in mice (169). MDR3 (mdr2) perform the function of transporting the phospholipids across the canalicular membrane into the bile duct (170; 171). Studies have shown that mutations in this transporter cause an inhibition of both phospholipid and cholesterol transport into the bile in mice (172). Studies have also reported that FC and phospholipid movement into the bile can be restored when the MDR3 gene is expressed in mdr2 knockout mice. These studies suggest that canalicular phospholipid transport is critical for the movement of FC into the bile (173). SR-BI is also found on the canalicular membrane and it has been proposed that it may play a role in FC transport across this apical membrane (152). Bile acids sorted in the hepatocyte are transported across the canalicular membrane into the bile canaliculus by a transporter, similar to MDR3, termed the sister gene to p-glycoprotein (sgpg) (156). Once in the bile canaliculus, the phospholipids and cholesterol form water-soluble vesicles that accumulate in the gall bladder until they are deposited into the intestines (156). The

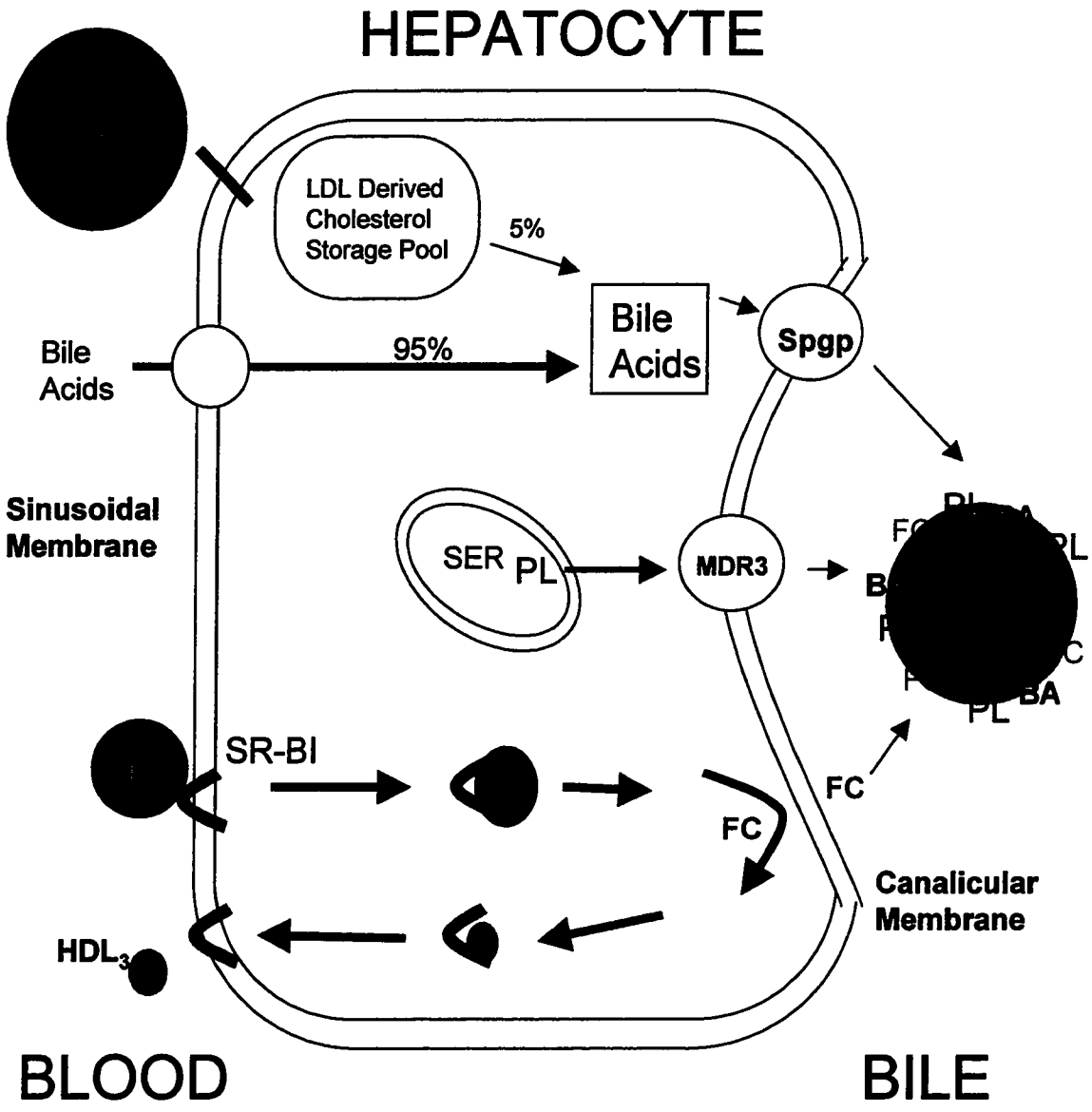
excretion of cholesterol into the bile has been reported to play an important role in regulating the proportion of dietary cholesterol absorbed by the body (174).

Studies have also shown that the over-expression of SR-BI can cause increased cholesterol transport and secretion into the bile (153). Reports made by Kozarsky *et al* (153) are consistent with earlier studies that suggested HDL-FC is the preferred type of cholesterol that is secreted in the bile (167; 175; 176) (Figure 3). Studies have also shown that the precursor cholesterol for bile acid synthesis is derived from the LDL and VLDL pools (177). Silver et al elucidated the transport of cholesterol via the SR-BI receptor using primary hepatocytes with a well-formed canalicular membrane region and polarized Madin-Darby canine kidney cells (152). This important study tracked the intracellular transport of HDL-cholesterol, HDL-protein, and SR-BI using cellular co-localization techniques. HDL is transported via a peri-nuclear vesicle to the canalicular region where the cholesterol from this vesicle is removed and delivered to the canalicular surface. HDL-protein is re-secreted on the basolateral side of the cell. This process well-describes the selective uptake pathway and provides evidence why HDL-FC is the preferred source of biliary cholesterol (152).

Cholesterol Reducing Drugs

Several drug therapies are currently being used to lower serum cholesterol levels and reduce the risk of developing atherosclerosis and heart disease. These drugs and agents affect various pathways in cholesterol metabolism. Some drugs, termed resins, reduce the dietary absorption of cholesterol by inhibiting the enterocyte uptake (178). Fibric acid derivatives have been administered extensively to reduce hepatic lipid synthesis and enhance VLDL catabolism (179) by acting as a peroxisome proliferator-

Figure 3. **Hepatic Transport of Cholesterol** Hepatocytes are polarized cells possessing a sinusoidal (contact with blood) and canalicular membrane (contacts bile canaliculus). The liver performs the function of filtering unwanted material into the intestines for disposal as well as synthesizing functional bile components. Bile acids (BA) are synthesized in the hepatocyte from the cholesterol precursor mainly from the LDL derived storage pool and transported through the canalicular membrane via BA transporter, sister p-glycoprotein (sgpg). The majority of the BA are recycled by the intestines through the portal vein to the liver where they are taken up by BA receptors and re-used. HDL derived cholesterol is taken up by the SR-BI receptor on the sinusoidal membrane and transported to the canalicular membrane in perinuclear recycling compartments. The cholesterol is delivered to the canaliculus through passive diffusion and absorbed by phospholipid vesicles. The transport of phospholipids into the bile canaliculus is essential for vesicle formation and the transport of cholesterol into the bile. This cholesterol transport is dependent on the action of the MDR3 transporter in the canalicular membrane. Phospholipid are synthesized in the smooth endoplasmic reticulum (SER) and then transferred to the canaliculus. Adapted from reference (156).


HEPATOCYTE
LDL Derived
Cholesterol
Storage Pool
5%
Bile
Acids
95%
Bile
Acids
Spgp
Sinusoidal
Membrane
SER PL
MDR3
SR-BI
FC
FC
Canalicular
Membrane
HDL3
BLOOD
BILE

activated receptor (PPAR alpha) ligand. Activation of PPAR alpha has been shown to decrease triglyceride plasma levels through increases in the expression of genes involved in fatty acid-beta oxidation, lower VLDL levels through increases in LPL expression and increase plasma HDL levels through increases in apoA-I expression (101). Other drugs, termed statins, inhibit HMG-CoA reductase, the enzyme involved in the rate-limiting step in cholesterol synthesis (180). The inhibition of this enzyme causes an up-regulation of the expression of the LDL-receptor on the surface of the liver, which results in an enhanced uptake of LDL-cholesterol and a decrease in serum levels (181-183). This has been shown to promote a significant reduction in the risk of developing atherosclerosis (184-186). Other drugs have been developed to inhibit the transfer of cholesterol to atherogenic lipoproteins such as LDL. A potent CETP inhibiting compound has been used in cholesterol fed rabbits and shown to reduce cholesterol levels and the development of atherosclerosis (131). CETP inhibition is believed to prevent the transfer of CE from HDL to VLDL and LDL.

The development of a drug that is specifically able to raise HDL-cholesterol and lower LDL-cholesterol would be a very powerful tool to prevent atherosclerotic progression (187). The use of the CETP inhibitor in rabbits resulted in a pronounced 3-fold increase in HDL-cholesterol levels and only a 50% decrease in total serum-cholesterol levels, however rabbits had a similar protection from atherosclerosis when compared to rabbits treated with statin drugs (131), indicating the importance of HDL in the prevention of atherosclerosis. Studies have also been performed to increase the reverse cholesterol transport pathway to the liver. One of these studies, performed in human volunteers, examined the effects of an injection of apoA-I into the blood stream

(188). This study showed that cholesterol output in the faeces increased after the injection, which indicated that apoA-I injection could stimulate reverse cholesterol transport. Another study showed that the injection of a large amount of phospholipid also increased the levels of FC in the serum and suggested that cholesterol efflux from cells and reverse cholesterol transport was being enhanced (189). The movement of cholesterol through any stage in the reverse cholesterol transport pathway is currently believed to be anti-atherogenic.

Rationale and Aims

As mentioned previously, atherosclerosis is one of the major causes of human death in the developed world despite major advances in research over the past 30 years. The primary goal of the research is to understand what factors regulate cholesterol metabolism in order to control the development of atherosclerosis. The literature suggests that the lipoprotein surface charge can regulate many of the interactions with proteins and enzymes that metabolise lipids (i.e., CETP, PLTP, LCAT, and SR-BI). The objective of this thesis is to characterise an alteration of lipoprotein charge on cholesterol metabolism using an *in vivo* and *in vitro* approach. The proposed protocol will utilise an intravascular injection of PI-vesicles in order to manipulate lipoprotein surface charge in rabbits. If the alteration of lipoprotein negative charge can enhance the clearance or movement of cholesterol from the serum to the liver for excretion, then it would suggest that this process might decrease the development of atherosclerosis and coronary heart disease.

EXPERIMENTAL PROCEDURE

Preparation of Phospholipid Vesicles

Isolated phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL). Phosphatidylcholine was isolated from bovine brain tissue extract, and was reported to contain mostly palmityl-oleoyl phosphatidylcholine (PC). PI was isolated from bovine liver extracts and contained a mixture of acyl chain length and saturation. The molecular weight of PI was based on the 18:0 - 20:4 acyl chain structure, although 18:0 - 18:1 was also reported to be present in the mixture. Phosphatidylserine (PS) lipids were isolated from bovine brain extracts and contained a mixture of acyl side chains length and saturation. The predominant acyl side chain isolated from the PS extract was reported to be 18:0 - 18:1. The characterisation of the phospholipid acyl-chain length and saturation was performed and reported by Avanti Polar Lipids.

PI, PS and PC vesicles were prepared by sonication. Specific amounts of phospholipids in chloroform were dried to completion under nitrogen in a 12 x 75 mm glass tube, at room temperature and 50 mM sodium phosphate, pH 7.2, 150 mM sodium chloride (PBS) (0.8 – 3 ml) was added. The lipid buffer mixture was sonicated for 1 minute at constant (100%) output while being cooled in a 10°C water bath. The mixture was incubated at 37°C for 15 minutes and then sonicated at a 90% output for 4 minutes while being cooled in a 10°C water bath, after which time the milky suspension became clear. Samples were centrifuged for 5 minutes at 3,000 g to remove any particulate titanium deposited from the sonicator probe. This technique was used to prepare phospholipid vesicle preparations of concentrations less than 8 mg/ml.

Animal Care

Male New Zealand white rabbits (Charles River, Montreal, QC), ~4.0 kg, were housed individually in metal cages with wire mesh bottoms in a room with controlled lighting (12 h/day) and constant temperature (18°C). Rabbits were removed from cages and petted every day for at least two weeks prior to the study to reduce the effects of stress from the experiment. Rabbits were maintained on normal chow diet (Charles River Rabbit Animal Diet, catalogue number 5079-17.5% protein, 2.8% fat, 25% fibre) and had free access to water.

Lipid Injections

Initial *in vitro* investigations reported that the addition of PI to human plasma (0.2 mg/ml) was sufficient to alter lipoprotein charge. It was estimated that a 4 kg rabbit would have ~180 ml of plasma based on the assumption that blood volume was ~9% of the body weight, and ~50% of the blood was plasma. Therefore, the injection of 36 mg of PI would represent an initial plasma concentration of 0.2 mg/ml. An equimolar concentration of PC lipid vesicle was prepared as a control. Rabbits were fasted for 12 hours prior to the experiment and remained fasted until after the final time point was taken. Rabbits had free access to water and were kept in their usual surroundings during the course of the study. A butterfly needle was inserted into the marginal ear vein and a pre-injection blood sample (2-6 ml) was collected. The vesicle solution of either 36 mg of PI (~9 mg/kg) or 31 mg of PC (~8 mg/kg) was injected into the marginal ear vein. A sample of blood (2-6 ml) was drawn at 10 and 30 minutes, 1, 3, 6, and 24 hour(s) after the injection of the lipid vesicles. All blood samples were drawn into tubes containing 7.5% (K_3) EDTA solution, immediately placed on ice and subsequently centrifuged at

3000 g for 15 minutes at 4°C to isolate the plasma. Plasma samples were stored for 4 days at 4°C and then transferred to a –80°C freezer. Less than 20% of the total blood volume was removed from the rabbit over a 24-hour period. The total volume of blood in the rabbit was assumed to be 9% of body weight. It was difficult to obtain a consistent level of blood from the rabbits due to variations in the size of the vein, blood flow, and bruising from repeated bleeds. In some circumstances, blood removal had to be stopped to ensure the subsequent time point was not affected.

To determine the rate of clearance of cholesterol from the rabbit plasma, a radioactive cholesterol tracer was added to the phospholipid vesicle preparation. Two hundred micro litres of 1 μCi/μl [1α, 2α-^{3}H]-FC (NEN, Boston, MA) in ethanol were dried in a 12 x 75 mm glass tube with PI or PC, and the lipid vesicles were prepared as described earlier. The phospholipid and tracer vesicles (6 ml) of either 36 mg of PI or 31 mg of PC were injected into the marginal ear vein. Blood was sampled from the opposite marginal ear-vein at previously described time points. Plasma was isolated from blood using the method described above.

Characterisation of Plasma Lipoproteins from Rabbits

Lipoprotein fractions were isolated from plasma by sequential ultracentrifugation. VLDL was isolated from the top of the plasma fraction at density = 1.019 g/ml after ultracentrifugation (6°C) at 417200 g for 3 hours. The bottom fraction was re-suspended and adjusted to a density = 1.063 g/ml, and LDL was isolated from the top fraction after ultracentrifugation (6°C) at 417200 g for 5 hours. Subsequently, this bottom fraction was re-suspended and adjusted to a density = 1.21 g/ml, and HDL was isolated from the top fraction after ultracentrifugation (6°C) at 181732 g for 15 hours. Densities were adjusted

by the addition of potassium bromide density solutions. See Table I for a summary of the lipoprotein densities. An Optima™ TLX Ultracentrifuge (Beckman Coulter, Palo Alto, CA), TLA 100.4 rotor (Beckman Coulter) and 5.1-ml sealed polyallomer bell-top tubes (Beckman Coulter) were used for all lipoprotein preparations. The isolated lipoproteins were dialysed overnight against PBS to remove potassium bromide. Lipoprotein lipid composition (total cholesterol, FC and TG concentrations) was determined using enzymatic assay kits from Roche Diagnostic (Laval, QC). The correlation coefficients were calculated from the standard curve for all assays and were routinely greater than 0.990 and therefore linear. All measured sample values were within the range of the standard curve. The CE values were determined from the difference between FC and total cholesterol measurements. The standard curve range for the FC and total cholesterol assays was 0.25 μg to 10 μg of cholesterol. The standard curve range for the TG assays used was 2.5 μg to 40 μg of TG. Protein concentrations were determined using the Lowry method as modified by Markwell *et al* (191). The standard curve range for the Lowry assay used was 2.5 μg to 40 μg of bovine serum albumin (BSA) (Sigma-Aldrich Canada Ltd, Oakville, ON). The correlation coefficients were calculated from the standard curve and were routinely greater than 0.990 and therefore linear. All measured sample values were within the range of the standard curve.

Surface charge characteristics of the lipoproteins were determined by electrophoresis on pre-cast 0.5% agarose gels (Beckman Coulter, Paragon Lipokit) according to the methods described by Sparks and Phillips (50). Briefly, electrophoresis was performed (4 μl of sample) for 30 minutes at 100 V in the kit barbital buffer (pH 8.6, 0.05 ionic strength). After electrophoresis, the gels were successively fixed in a solution

of ethanol-acetic acid-water 6:1:3 (v/v/v), dried, and stained for 5 minutes with a 0.07% solution of Sudan Black B in ethanol-water 11:9 (v/v). Gel portions containing purified apoA-I, which was used as an internal standard, were stained with a 0.8 g/l solution of Coomassie Brilliant Blue G 250 in 0.33 M perchloric acid, and de-stained in a solution of methanol-acetic acid-water 7:5:8 (v/v/v). Electrophoretic mobility (U) was determined as previously described by Sparks *et al*, by dividing the electrophoretic velocity (migration distance (μm)/time (s)) by the electrophoretic potential (voltage (V)/gel distance (cm)). To correct for the isoelectric point-dependent retardation effects, the following equation was applied (50):

$$U_{corrected} = (U_{agarose} - 0.136)/1.211 \qquad \textit{Eq. 1)}$$

The surface potentials (S) of the lipoproteins were estimated as previously described by Sparks *et al,* from $U_{corrected}$ using the most general form of Henry's equation (50):

$$S = U6\Pi n/D \qquad \textit{Eq. 2)}$$

where D is the solvent dielectric constant, and n is the coefficient of viscosity.

Concentrations of PI in the isolated lipoproteins were determined by thin-layer chromatography (TLC). Total lipids of the lipoproteins were obtained by chloroform-methanol 2:1 (v/v) extraction (243). The chloroform phase was dried to completion under nitrogen and dissolved in a fixed amount of chloroform. The total lipid extracts were separated on silica gel plates using a solvent system composed of chloroform-methanol-ammonia, 13:7:1 (v/v/v). Plates were charred with a 10% copper sulphate (w/v), 8% phosphoric acid (w/w) solution. PI concentration was determined from PI standard curve by densitometry, using Bio-Rad-Quantity-One (version 4.03) software. The PI values were expressed as a ratio with the original protein levels associated with

the isolated lipoproteins. The standard curve values ranged from 0.25 μg to 10 μg. The correlation coefficient from the standard curve was 0.990 and therefore linear. Samples were only deemed detectable when the values were found within the range of the standard curve. Protein concentrations were determined using the Lowry method as modified by Markwell *et al* (191).

Measurement of Rabbit Plasma and Lipoprotein Radioactivity

Plasma samples (20 μl), from rabbits injected with [^{3}H]-FC, were combined with EcoLite scintillation cocktail (ICN, Costa Mesa, CA) (4 ml), vortexed and measured for Beta emission by a TRI-CARB 2100TR Liquid Scintillation Analyser (Canberra Packard, Mississauga, ON). In addition, the [^{3}H]-FC was separated from the newly formed [^{3}H]-CE in the isolated lipoprotein samples using TLC. Ethanol (2 ml) was added to lipoprotein (20 μl) aliquots, and incubated for 2 hours at room temperature. The supernatant was collected after centrifugation (10 minutes at 10,000 g), incubated with hexane carrier (containing 50 μg/ml of CE and 50 μg/ml of FC) and then evaporated to dryness under nitrogen. The samples were dissolved in chloroform (200 μl), spotted (50 μl) on TLC plates (Silica Glass Fiber, Gelman Science, Ann Arbor, MI), run in hydrophobic solvent system containing hexane, diethyl ether and acetic acid, 89:10:1 (v:v:v). FC and CE were visualized with iodine staining. Bands corresponding to FC and CE were cut and radioactivity was determined. The total counts in each circulating lipoprotein fraction were derived from the plasma volume correction.

Measurement of Rabbit Plasma Cholesterol Esterification Rate

Cholesterol esterification rates of plasma samples from PI and PC injected rabbits were analysed as previously described (192). Plasma samples (400 μl) were incubated

with [^{3}H]-FC on filter paper discs (10 μCi of [^{3}H]-FC) at 4°C overnight. From each sample, three aliquots (50 μl) were removed and used as the time zero point, and three aliquots (50 μl) were incubated at 37°C for 30 minutes. Ethanol (2 ml) was then added to the time 0 and 30-minute samples and incubated for 2 hours at room temperature. The supernatant was collected after centrifugation (10 minutes at 10,000 g), incubated with hexane carrier (containing 50 μg/ml of CE and 50 μg/ml of FC) and then evaporated to dryness under nitrogen. The samples were dissolved in chloroform (200 μl), and 50 μl was spotted on TLC plates (Silica Glass Fiber Gelman Science, Ann Arbor, MI). The plates were run in a hydrophobic solvent system containing hexane-diethyl ether-acetic acid, 89:10:1, (v:v:v). FC and CE bands were visualised with iodine staining. Bands corresponding to FC and CE were cut and radioactivity was determined. The fractional esterification rate is expressed as the percentage of FC converted to CE per hour.

Enrichment of Human and Rabbit Plasma Lipoproteins with Phospholipids In vitro

Human plasma was obtained from a fasting male normal-lipidemic volunteer. Rabbit plasma was obtained from fasted rabbits. HDL was isolated from human plasma by sequential ultracentrifugation. VLDL, LDL was isolated from the top of the plasma fraction at density = 1.063 g/ml after ultracentrifugation (6°C) at 417,200 g for 5 hours. Subsequently, the bottom fraction was re-suspended and adjusted to a density = 1.21 g/ml, and HDL was isolated from the top fraction after ultracentrifugation (6°C) at 181,732 g for 15 hours. Densities were adjusted by the addition of potassium bromide density solutions. An Optima™ TLX Ultracentrifuge (Beckman Coulter, Palo Alto, CA), TLA 100.4 rotor (Beckman Coulter), and 5.1-ml sealed polyallomer bell-top tubes (Beckman Coulter) were used for all lipoprotein preparations. The isolated lipoproteins

were dialysed overnight against PBS to remove potassium bromide. Protein concentrations of HDL were determined using the Lowry method as modified by Markwell *et al* (191). PI-, PC-vesicles, or PBS was incubated with the isolated HDL (0.2 mg/mg of HDL) for 24 hours at 4°C. HDL surface charge characteristics were determined by electrophoresis on pre-cast 0.5% agarose gels (Beckman, Paragon Lipokit) (50) as described above. PI-, PC-vesicles or PBS was incubated with the human and rabbit plasma (1.0 mg/ml plasma) for 48 hours at 4°C *in vitro*. Cholesterol esterification rates of the plasma samples were determined by using the method described above. PI-, PC- or PS-vesicles were incubated with the human plasma (0.2 or 0.4 mg/ml plasma) for 1 or 16 hour(s) at 37°C *in vitro*. The plasma lipoprotein surface charge characteristics were determined by electrophoresis on pre-cast 0.5% agarose gels (Beckman, Paragon Lipokit) (50). In some experiments, HDL and LDL were ultra-centrifugally isolated from the plasma (as described above) dialysed against PBS and then subjected to electrophoresis on the pre-cast 0.5% agarose gels.

Lipid Vesicle Injections, Enriched HDL Injections and [^{3}H]-FC in the Liver and Bile

In some experiments, rabbits were euthanised 30 minutes post injection of [^{3}H]-FC (0.5 mCi) and PI- (36 mg) (n = 1) or PC- (31 mg) (n = 1) vesicles. The rabbits were sedated with fentanyl citrate and fluanisone (70 μg/kg and 2.2 μg/kg intramuscularly, respectively) 20 minutes following the injection of the phospholipid lipid. Thirty minutes following the lipid injection, a lethal dose was administered (pentobarbital, 100 mg/kg). The abdominal cavity was then opened by a ventral incision and the liver and gall bladder were exposed. The common bile duct was ligated with silk thread and bile was aspirated from the gall bladder with a catheter and syringe. The total volume of bile was measured.

Aliquots of bile (5 µl) were added to Eco-Lite scintillation cocktail (ICN) (4 ml), vortexed and measured for beta emission using a TRI-CARB 2100TR Liquid Scintillation Analyser (Canberra Packard, Mississauga, ON). The total radioactive counts in the bile were calculated from total volume. The liver was removed, rinsed with saline, and weighed. Three samples (1 gram each) were taken from three different regions of each liver and homogenised in water (2 ml). The total lipids were extracted from the liver homogenate as described by Bligh and Dyer (194). A chloroform and methanol solution (7.5 ml), 1:2 (v:v), was added to the homogenised tissue and the mixture was vortexed. Chloroform (2.5 ml) was added to the mixture and vortexed. Water (2.5 ml) was added to the mixture and vortexed. Mixture was centrifuged at 2000 g for 10 minutes to separate the phases. The bottom (organic) phase was dried under a nitrogen stream. The residue was mixed with 4 ml of BetaMax scintillation cocktail (ICN), vortexed heavily and measured for beta emissions using a TRI-CARB 2100TR Liquid Scintillation Analyser (Canberra Packard, Mississauga, ON) 24 hours later. Total radioactive counts from the liver were derived from the total weight of the organ and the average counts from the extracted sample.

To understand the role that HDL plays in the PI-enhanced FC clearance in rabbits, an injection of phospholipid-enriched HDL into rabbits was also performed. Fasting rabbit plasma was incubated with 0.2 mg/ml of PI or PC vesicles for 3 days at 4°C. VLDL and LDL were isolated from the top of the plasma fraction at density = 1.063 g/ml after ultracentrifugation (6°C) at 417200 g for 5 hours. Subsequently, this bottom fraction was re-suspended and adjusted to a density = 1.21 g/ml, and HDL was isolated from the top fraction after ultracentrifugation (6°C) at 181732 g for 15 hours. The HDL

was dialysed overnight against PBS to remove potassium bromide. Both PI- and PC-enriched HDL was radioactively labelled through incubation with PC-vesicles prepared with [^{3}H]-FC. HDL was incubated with [^{3}H]-FC/PC vesicle complex (0.3 mCi) at 4°C for 48 hours to label the lipoprotein. PI-HDL (2 mg) (n = 1) and PC-HDL (2 mg) (n = 1) was injected into the rabbits through the marginal ear vein as described above. The rabbits were sedated with an intramuscularly injection of fentanyl citrate and fluanisone (70 µg/kg and 2.2 µg/kg, respectively) 20 minutes following the injection of the HDL. Thirty minutes following the lipid injection, a lethal dose was administered (pentobarbital, 100 mg/kg). The radioactivity associated with the bile and liver was determined as described above.

Lipid Injections and Cholesterol and Bile Acid Levels in the Faeces

Faeces from rabbits were collected daily for 6 days, following injection of PI (36 mg) (n = 2) or PC (31 mg) (n = 2) vesicles. To ensure constant daily faecal output over the six days, rabbits were allowed free access to food and water. Faeces were stored at -80°C. All faeces were lyophilised overnight and the total daily samples were weighed to determine dry mass output for each day. Daily samples were mixed, and three random faecal samples (100 mg each) were removed from each day and were placed in a separate test tube. Total lipids and bile acids were extracted from the sample using the technique described by de Wael *et al* (195). Briefly, ethylene glycol in KOH (2 ml) was added to the sample and heated for 20 minutes at 210°C. Solution was then cooled to room temperature and concentrated hydrochloric acid (200 µl) was added. Diethyl ether (10 ml) was added, the solution was vortexed and then centrifuged for 15 minutes at 2000 g, followed by the removal of the upper phase of the two-phased solution. The mixture was

washed three more times with diethyl ether (10 ml). The pooled diethyl ether (40 ml) was dried under nitrogen at 37°C and the residue was dissolved in methanol (1 ml). In order to test for the percent recovery of faecal cholesterol from the extraction procedure, [^{3}H]-FC was added to the ground faecal sample prior to extraction. The recovery of the exogenously added [^{3}H]-FC was usually greater than 90%. Total cholesterol and bile acids in the faecal extracts were assayed with enzymatic kits purchased from Roche Diagnostics and SIGMA Diagnostics (St. Louis, MO), respectively. The range of the standard curve used for the total cholesterol assay was 0.25 μg (0.7 nmol) to 20 μg (56 nmol) of cholesterol. The correlation coefficient for the standard curve used in the cholesterol assay was calculated as 0.99 and therefore linear. All measured sample values were within the range of the standard curve. The range for the standard curve used for the total bile acid assay was 2.5 μg (5.8 nmol) to 40 μg (93 nmol) of cholate standard. The correlation coefficient for the standard curve used in the total bile acid assay was calculated as 0.99 and therefore linear. All measured sample values were within the range of the standard curve.

Cell Culture

The human hepatoma cells, HepG2 (196), were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin sulphate. Chinese hamster ovary (CHO) cell lines that lack a functional LDL receptor (ldlA7) and ldlA7 cells stably transfected with recombinant SR-BI (ldlA7-SRBI) were used in this study (197; 198). The stably transfected ldlA7-SRBI cells were a generous gift from Dr. Helen Hobbs (University of Texas South-western Medical Center at Dallas, Dallas, Texas). The

CHO cells were maintained in Ham's F12 medium supplemented with 10% (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin sulphate. The ldlA7-SRBI cells were supplemented with Geneticin® (G418 sulfate) to select the positive transfectants. All cells were seeded at 1 x 10^6 in a 10 ml/10-mm Petri dish. All cells were cultured at 37°C, under 5% CO_2. All cell culture reagents and plastic-ware were purchased from either GIBCO/BRL (Grand Island, NY) or Becton Dickenson and Company (Franklin Lakes, NJ).

Plasma and Lipoprotein Preparations for Cell Experiments

Human plasma from male normal lipidemic fasting volunteers was incubated with PI (0.2 mg/ml) or PC (0.2 mg/ml) vesicles at 4°C for 24 hours. Plasma total cholesterol, FC concentrations were determined using enzymatic assay kits from Roche Diagnostic (Laval, QC). The CE was calculated from the difference between FC and total cholesterol measurements as described above. Plasma was also simultaneously incubated and labelled with [^{3}H]-FC dried on filter discs (5 μCi/ml plasma) at 4°C for 24 hours. To examine CE-uptake, some plasma was incubated with PC- or PI-vesicles (0.2 mg/ml) that were prepared with [1α, 2α-^{3}H]-CE (NEN) (10 μCi/ml of plasma). Labelled plasma was added to EMEM (5% (v/v)) containing BSA (2 mg/ml) and filtered through a sterile 0.2-μm filter (Millipore Corporation, Bedford, MA).

Human HDL and LDL were isolated from plasma obtained from male normal-lipidemic fasting volunteers. VLDL was isolated from the top of the plasma fraction at density = 1.019 g/ml after ultracentrifugation (6°C) at 417200 g for 3 hours. The bottom fraction was re-suspended and adjusted to a density = 1.063 g/ml, and LDL was isolated from the top fraction after ultracentrifugation (6°C) at 417200 g for 5 hours.

Subsequently, this bottom fraction was re-suspended and adjusted to a density = 1.21 g/ml, and HDL was isolated from the top fraction after ultracentrifugation (6°C) at 181732 g for 15 hours. Densities were adjusted by the addition of potassium bromide density solutions. An Optima™ TLX Ultracentrifuge (Beckman Coulter, Palo Alto, CA), TLA 100.4 rotor (Beckman Coulter) and 5.1-ml sealed polyallomer bell-top tubes (Beckman Coulter) were used for all lipoprotein preparations. The isolated HDL and LDL were dialysed overnight against PBS to remove potassium bromide. Protein concentrations of HDL and LDL were determined using the Lowry method as modified by Markwell *et al* (191). HDL- and LDL-FC concentrations were determined using enzymatic assay kits from Roche Diagnostic (Laval, QC). HDL or LDL was incubated with PI (0.2 mg/mg) or PC (0.2 mg/mg) vesicles or PBS at 4°C for 24 hours. HDL or LDL was also simultaneously incubated and labelled with [^{3}H]-FC dried on filter discs (5 μCi/mg protein) at 4°C for 24 hours. The specific activities of the lipoproteins were derived from the average radioactivity measurements and mass of HDL-FC or LDL-FC. HDL or LDL was added to EMEM or F12 medium (50 μg/ml), containing BSA (2 mg/ml) and filtered through a sterile 0.2-μm filter (Millipore Corporation).

In other studies HDL was radio-iodinated using Iodo-Beads® (Pierce, Rockford, IL). Briefly, the Iodo-Beads® (Pierce) were washed in PBS and incubated with ^{125}I (2 mCi of NaI) in 70 μl of PBS for 5 minutes at room temperature. An aliquot (40 μl of a 1mg/ml solution) of HDL was then added to the reaction mixture and incubated at room temperature for 45 minutes with continuous agitation. In order to remove the un-incorporated free iodine, the mixture was then passed through a desalting column (Excellulose GF5, Pierce) previously equilibrated with PBS and 1% BSA, and the eluate

was extensively dialysed against PBS. The HDL specific activity value was derived from the average radioactivity measurements and mass of HDL.

Cell Culture Experiments

All cells were plated into 24 well tissue culture dishes in complete medium. On day 3, medium was removed and cells were washed twice with FBS free medium. For uptake studies, the lipoprotein/medium mixture (1 ml) was added to each well and was incubated for various amounts of time. Following the incubations, the cells were placed on ice and the medium was removed. The cell monolayer was washed twice with cold PBS containing BSA (2 mg/ml) and twice with cold PBS. Cells were solublised in 1 ml of 0.1 M NaOH on a plate rocker overnight at room temperature. In experiments where a tritium tracer was added to the cells, a fraction (0.7 ml) of cell lysate was mixed with Eco-Lite scintillation cocktail (ICN) (3.5 ml), vortexed and the cell-associated radioactivity was measured using a TRI-CARB 2100TR Liquid Scintillation Analyser (Canberra Packard, Mississauga, ON). In the experiment where ^{125}I labelled HDL was used, the radioactivity in the cell lysates (0.7 ml) was measured directly in a Cobra II Auto-Gamma counter (Canberra Packard). Total cell protein was determined using a BCA enzymatic assay kit (Pierce) (200). The standard curve range for this assay was 0.25 μg to 40 μg of BSA. The correlation coefficients were calculated from the standard curve and were routinely greater than 0.990 and therefore linear. All measured sample values were within the range of the standard curve.

Cellular cholesterol uptake experiments were performed in the presence or absence of cell signalling modulators. Cells were washed three times in medium containing BSA (2 mg/ml) and then incubated in medium containing BSA (2 mg/ml)

with or without modulators at 37°C for 1 hour, prior to the addition of HDL. PI-vesicles (10 μg/ml medium) were added to pre-treatment mixture. Propranolol (200 μM) (Sigma-Aldrich Canada Ltd, Oakville, ON) was used as a specific inhibitor of the phosphatidic acid-phosphohydrolase (PAPH) activity. Chelerythrine chloride (10 μM) (Sigma-Aldrich) was used as a specific inhibitor of the protein kinase C (PKC). Phorbol 12-myristate 13-acetate (PMA) (40 μM) (Sigma-Aldrich) was used to stimulate PKC.

Statistical Analysis

The significance of difference between two means was calculated using a two-tailed Student's *t*-Test for un-paired data. $P < 0.05$ was regarded as statistically significant. A one-way ANOVA was used to determine significance of difference between multiple group means. If $P < 0.05$, Post Hoc analyses were performed using the Tukey Kramer Multiple Comparison Test. $P < 0.05$ was regarded as statistically significant.

RESULTS

The Effects of Phospholipid Vesicles on Lipoprotein Charge in vitro

The surface potential of lipoprotein particles in human plasma incubated with (0.2 mg/ml) PI-, PC- or PS-vesicles for 1 hour at 37°C was determined by agarose gel electrophoresis. The plasma incubated with PS- or PI-vesicles displayed an increased electrophoretic-migration (increased surface potential) of three classes of lipoproteins when compared to PC-enriched plasma (Table II). However, statistical analysis showed that the LDL from the plasma incubated with the PS-vesicles was not significantly different from that of the LDL from the PC-enriched plasma. The surface charge of HDL and VLDL appeared to be more sensitive to the incubation with PI and PS than LDL at these conditions.

Figure 4 compares HDL and LDL isolated from human plasma that was incubated in the absence or presence of 0.4 mg/ml PI-vesicles at 37°C for 16 hours. HDL and LDL from the PI-enriched plasma have a greater negative surface potentials (-18 mV and -10 mV, respectively) than the HDL and LDL isolated from the control plasma (-10 mV and -4 mV, respectively). This suggested that the anionic lipids were absorbed into the outer surface lipid layer of lipoproteins and caused the plasma constituents to become more negatively charged. This effect of PI on surface charge appeared to be dose and time dependent as the HDL and LDL had a greater negative surface charge when compared to the results in Table II.

Figure 4. **The effects of PI on HDL and LDL surface charge in plasma.** Human plasma was incubated in the absence or presence of PI-vesicles (0.4 mg/ml) at 37°C for 16 hours. HDL and LDL were separated ultra-centrifugally from PI-enriched and untreated plasma. Samples were subjected to electrophoresis in pre-cast 0.5% agarose gels and surface potential ± 0.3 (-mV) was calculated as described in the Experimental Procedures.

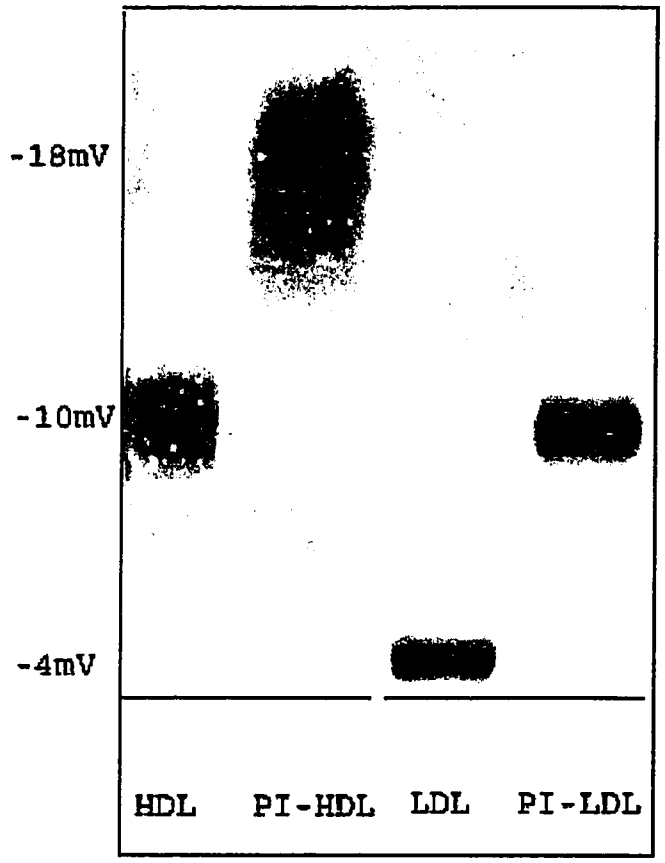

-18mV
-10mV
-4mV
HDL
PI-HDL
LDL
PI-LDL

Table II. The effects of lipid vesicle incubations with human plasma on lipoprotein surface charge. [a]

Lipid Vesicle	Surface Potential (-mV) [b]
PC (n = 3)	
HDL	11.8 ± 0.3
LDL	4.3 ± 0.2
VLDL	7.4 ± 0.3
PI (n = 3)	
HDL	** 14.1 ± 0.1
LDL	* 5.0 ± 0.3
VLDL	** 10.0 ± 0.5
PS (n = 3)	
HDL	** 13.2 ± 0.1
LDL	4.8 ± 0.2
VLDL	** 9.1 ± 0.3

[a] Plasma from a normal lipidemic fasting male volunteer was incubated with phospholipid vesicles (0.2 mg/ml) for 1 hour at 37°C. Plasma samples were subjected to electrophoresis in precast 0.5% agarose gels and surface potential of each lipoprotein was calculated as described in the Experimental Procedures.

[b] The values represent the mean ± S.D. surface potential in –mV from three independent lipid vesicle incubations with plasma.

A one-way ANOVA analysis was performed and determined significant variance between the three groups of lipid enriched plasma lipoproteins. HDL ($F(2,6) = 133.7$, $P < 0.0001$), LDL ($F(2,6) = 6.7$, $P < 0.05$) and VLDL ($F(2,6) = 33.2$, $P < 0.001$). Post hoc analysis using the Tukey Kramer Multiple Comparison test was also performed to compare mean values to the PC incubated control (* $P < 0.05$, ** $P < 0.01$).

Isolated human HDL molecules incubated with PI-vesicles (0.2 mg/mg HDL) for 24 hours at 4°C, significantly increased the negative surface potential when compared to normal isolated HDL or to HDL that had been incubated with an equivalent mass of PC-vesicles (Table III). There was no significant difference between HDL and PC-enriched HDL.

Table III. The effects of PI vesicles incubation on HDL surface charge *in vitro*.

	Lipid[a] (mg/mg)	Incubation Time (h)	Incubation Temperature (°C)	Surface[b] Potential (-mV)
HDL (3)	0	NA	NA	11.4 ± 0.3
HDL + PC (3)	0.2	24	4	11.5 ± 0.3
HDL + PI (3)	0.2	24	4	*13.3 ± 0.3

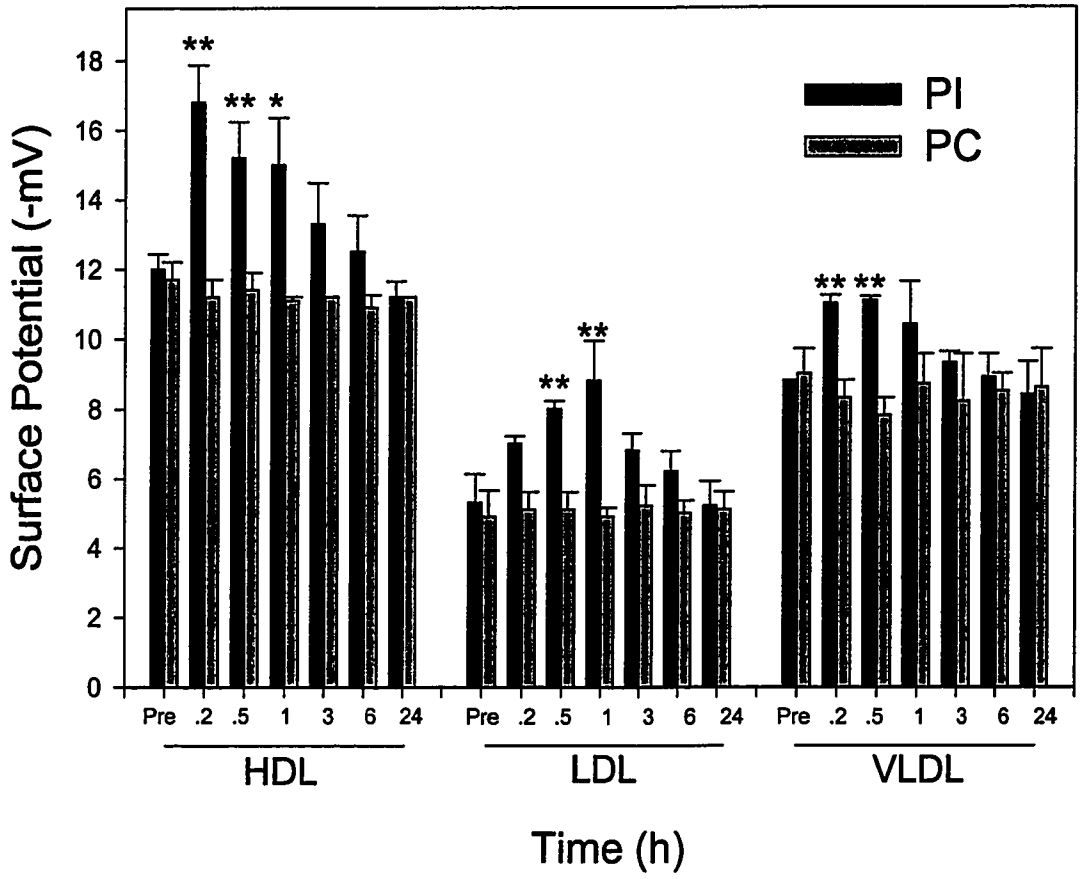
[a] Isolated HDL from a normal lipidemic fasting male volunteer was incubated with phospholipid vesicles at various ratios.
NA, not applicable.
[b] The values represent the mean ± S.D. surface potential in –mV of three independent preparations of HDL, HDL + PI and HDL + PC.
One-way ANOVA analysis determined there to be significant variance ($F(2,6) = 44.3$, $P < 0.003$) between the three different mean surface potentials. Post hoc analysis using the Tukey Kramer Multiple Comparison test indicated that the surface potential of HDL + PI is significantly different than that of HDL and HDL + PC (* $P < 0.001$).

The Effects of PI-Vesicle Injection on Lipoprotein Charge in Rabbits

Although, PS- and PI-vesicles were both shown to alter the surface charge of lipoproteins *in vitro*, PI was chosen for the following rabbit studies. PI is the most prevalent anionic phospholipid in the plasma and HDL. An injection of PS vesicles was more complicated because of the role that PS plays in the triggering of the blood coagulation cascade. All of the *in vivo* experiments described herein represent studies that I set up and performed. Repeat experiments have been performed by research staff in the laboratory of Dr. Sparks, and will not be described in this thesis. The work has led to the development of a novel cholesterol lowering therapy that is patent pending.

Figure 5 illustrates the effects of PI- and PC-vesicle injections in rabbits, on the estimated surface potential of HDL, LDL, and VLDL at various times points. While injection of PC ($n = 2$) had no obvious effect on lipoprotein charge, injection of PI-vesicles ($n = 4$) caused all lipoprotein fractions, at early time points, to migrate further on

Figure 5. **The effects of PI- or PC-vesicle injections on lipoprotein surface charge in rabbits.** Rabbits were injected with either PI- (36 mg) or PC- (31 mg) vesicles. Plasma samples were taken at various time points. Lipoproteins were isolated ultra-centrifugally and lipoprotein surface potentials were determined as described in the Experimental Procedures. Values represent the average (range) of two different rabbit PC-injections or the mean ± S.D. of four different rabbit PI-injections. A one-way ANOVA was performed and revealed that there was a high degree of significance of variance in the PI-injected rabbits for HDL ($F(6, 21) = 13.2$, $P < 0.0001$), LDL ($F(6, 21) = 11.8$, $P < 0.0001$), and VLDL ($F(6, 21) = 8.9$, $P < 0.0001$) surface charge values. Post hoc analysis using the Tukey Kramer Multiple Comparison test was performed and comparisons were made to the pre-injection values (* $P < 0.05$, ** $P < 0.01$).


Surface Potential (-mV)
0
2
4
6
8
10
12
14
16
18
**
** *
**
**
** **
PI
PC
Pre .2 .5 1 3 6 24
Pre .2 .5 1 3 6 24
Pre .2 .5 1 3 6 24
HDL
LDL
VLDL
Time (h)

the 0.5 % agarose gel, indicative of an increased negative surface charge (Figure 5). The increased negative charge in the PI-injected rabbit reached a maximum level early in the time course and then returned to normal values by 24 hours. The HDL fraction exhibited an initial surface potential of –12.2 mV, prior to the PI-injection and then reached a peak negative charge of –16.8 mV, 10 minutes after the PI-injection. Similarly, the VLDL fraction had an initial negative surface potential of –8.8 mV, which increased to -11.0 mV after 10 minutes. LDL surface charge peaked later; LDL had an initial negative surface potential of –5.3 mV, and peaked at –8.8 mV, 60 minutes after the PI-injection. Although the lipoprotein surface charges were averaged from only two PC-injected rabbits, it appeared that there was no apparent change in this parameter over the time course. A significant difference was observed between the pre-injection and post-injection mean surface potential values from the PI-injected rabbits (Figure 5). This data therefore indicated that an injection of PI-vesicles significantly altered the surface potential of all classes of lipoproteins in rabbits.

The Effects of PI-Vesicle Injections on Lipoprotein Composition in vivo

Plasma lipoprotein compositions of ultra-centrifugally isolated lipoproteins sampled before and after the bolus injection of phospholipids were determined. The protein concentrations of HDL, LDL and VLDL fractions remained relatively constant after the injection of PI-vesicles (n = 4) (Table IV). The HDL and LDL fractions exhibited no obvious change in TG/protein, CE/protein, or FC/protein ratios after injection of PI. A one-way ANOVA was performed on all lipoprotein lipid and protein values from the PI-injected rabbits. The results from this analysis of variance indicated that variations in the mean protein and lipid levels at the different time points were not

significantly greater than expected by chance. VLDL fractions from the PI-injected rabbit appeared to increase in CE/protein and FC/protein ratios after 1 and 3 hours when compared to initial pre-injection values, however as mentioned previously, no significance was observed. An experiment performed with a larger number of rabbits would be desirable to confidently report changes in lipid levels. In some circumstances, blood volumes were insufficient to acquire values for lipoprotein TG composition.

Conclusions from the lipoprotein compositional values of the PC-injected rabbits (n = 2) in Table IV cannot be made due to large variations between the lipid levels of the two PC-injected rabbits. The mean lipoprotein composition values from the two individual PC-injected rabbits were reported in Appendix 1. These reported means and standard deviation from each individual PC-injected rabbit indicated that the variations associated with the assay measurement were in most cases less than 10%. The means for the lipid/protein ratios were calculated from the protein and lipid values and therefore combined the S.D. values from each measurement. The combined error may account for some of the variations observed between rabbits, however, because the large variations between rabbits were greater than 10%, these variations are more likely due to true rabbit differences and not measurement error. One concern with Table IV was the relatively large variations in lipoprotein lipid values between PI-injected rabbits, which resulted in a large standard deviation and standard error of the mean. Studies performed by Whitlock *et al* utilized a similar technique to measure the lipoprotein lipid and protein concentrations in New Zealand white rabbits (85). This group reported a similar large variation (with n = 8) for each class of lipoproteins compared to values reported in Table IV.

Table IV. The effects of lipid injection on lipoprotein lipid and protein composition †

	Time h	CE/Protein[a] mg/mg	FC/Protein[a] mg/mg	TG/Protein[b] mg/mg	Protein[c] mg/dL
HDL					
PC	0	0.33 (0.20)	0.067 (0.014)	0.32 (0.40)	68 (22)
	1	0.25 (0.14)	0.079 (0.017)	0.27 (0.30)	59 (21)
	3	0.23 (0.19)	0.058 (0.035)	0.22 (0.12)	54 (21)
	24	0.30 (0.37)	0.062 (0.024)	0.41 (0.40)	63 (32)
PI	0	0.32 ± 0.14	0.10 ± 0.02	0.17 (0.24)	43 ± 12
	1	0.29 ± 0.15	0.11 ± 0.04	0.23 (0.24)	40 ± 4
	3	0.26 ± 0.12	0.08 ± 0.03	0.22 (0.21)	45 ± 7
	24	0.34 ± 0.20	0.11 ± 0.04	0.38 (0.36)	42 ± 10
LDL					
PC	0	1.2 (0.5)	0.50 (0.50)	2.3 (0.6)	2.9 (2.4)
	1	1.2 (0.1)	0.96 (0.53)	2.1 (0.4)	2.2 (1.5)
	3	1.37[d]	0.87 (0.05)	2.4 (0.4)	1.8 (0.8)
	24	4.2 (3.2)	0.75[d]	3.4 (0.6)	3.6 (1.0)
PI	0	0.92 ± 0.25	0.46 ± 0.20	1.8 (1.0)	6.0 ± 1.7
	1	1.10 ± 0.42	0.53 ± 0.25	1.7 (0.7)	4.9 ± 1.4
	3	0.84 ± 0.26	0.48 ± 0.21	1.6 (0.8)	5.7 ± 1.3
	24	1.83 ± 1.22	0.76 ± 0.27	3.3 (2.0)	6.2 ± 1.7
VLDL					
PC	0	0.44 (0.44)	0.20 (0.10)	3.1 (3.1)	6.5 (1.0)
	1	0.47 (0.19)	0.28 (0.15)	3.4 (3.1)	6.5 (3.0)
	3	0.45 (0.03)	0.33 (0.32)	4.0 (1.2)	6.0 (0.1)
	24	0.61 (0.17)	0.50 (0.25)	6.0 (4.0)	8.0 (2.0)
PI	0	0.43 ± 0.13	0.17 ± 0.10	1.7 (0.5)	5.2 ± 1.3
	1	1.0 ± 0.05	0.41 ± 0.13	2.2 (0.9)	5.7 ± 0.9
	3	1.2 ± 0.20	0.60 ± 0.17	5.6[d]	5.2 ± 1.0
	24	0.69 ± 0.22	0.38 ± 0.11	2.5 (2.6)	8.0 ± 1.8

† HDL, LDL and VLDL from PI (n = 4) and PC (n = 2) injected rabbits were assayed for triglyceride (TG), cholesterol (FC), total cholesterol and protein. CE was calculated from the difference between FC and total cholesterol.

[a] CE/Protein and FC/Protein values represent the mean ± SEM or average (range) in mg of lipid/mg HDL, LDL or VLDL protein. The range is expressed in parentheses.

[b] TG/protein values represent average (range) from 2 injections of PI or PC expressed in mg of TG/mg HDL, LDL or VLDL protein.

[c] Protein values represent the mean ± SEM (when n = 4) or average (range) (when n = 2) in mg/dl plasma.

[d] Single determination only

ANOVA was performed on the mean values from the PI injected rabbits. No significant variance was determined to justify further post hoc analysis.

The lipoproteins from one PI-injected animal were isolated and the PI mass was approximated by the TLC technique. The PI compositions in HDL and LDL increased from being below detectable levels to 0.062 and 0.32 mg/mg lipoprotein, respectively, after 10 minutes post-injection (Table V). PI levels in HDL decreased to undetectable levels by 3 hours. The levels of PI in LDL dropped to below detectable levels by the 30-minute time point. This experiment should be repeated to be validated. The sensitivity of the assay would also need to be increased in order to evaluate conclusive changes in lipoprotein PI levels following PI-injection. Prusanski *et al* reported detectable levels of PI in normal human HDL (Table V) using normal-phase liquid chromatography/mass spectrometry technique (84).

Table V. The effect of a PI-injection on lipoprotein PI composition in rabbits

Time Post Injection (h)	PI/HDL [a] (mg/mg)	PI/LDL [a] (mg/mg)	Human PI/HDL [b] (mg/mg)
0	ND	ND	0.013 ± 0.002
0.2	0.062 (0.003)	0.32 (0.02)	
0.5	0.037 (0.002)	ND	
1.0	0.037 (0.002)	ND	
3.0	ND	ND	

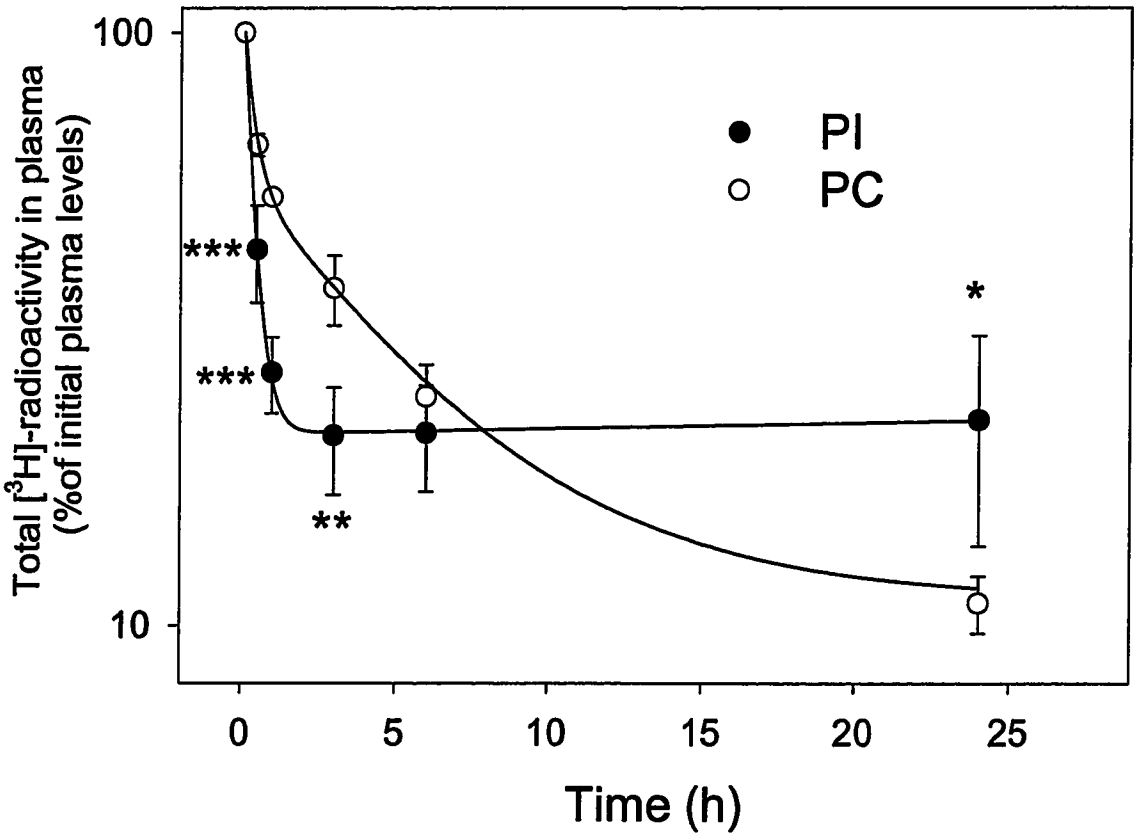
[a] HDL and LDL at several time points from one PI (36 mg) injected rabbit were assayed for PI and protein. Values represent an average (range) from duplicate measurements and are expressed as mg of PI per mg HDL or LDL protein.
ND; not detected.
[b] PI levels in normal human HDL (mg/mg HDL protein) adapted from ref. 84 and value represents the mean ± S.D. (n = 4).

The Effects of PI-Injection on the Clearance of [^{3}H]-Tracer from Plasma in Rabbits

Figure 6 compares the clearance of total [^{3}H]-tracer (^{3}H-FC and ^{3}H-CE) at each time point in rabbits injected with PI-vesicles (n = 3) or PC-vesicles (n = 3). The initial

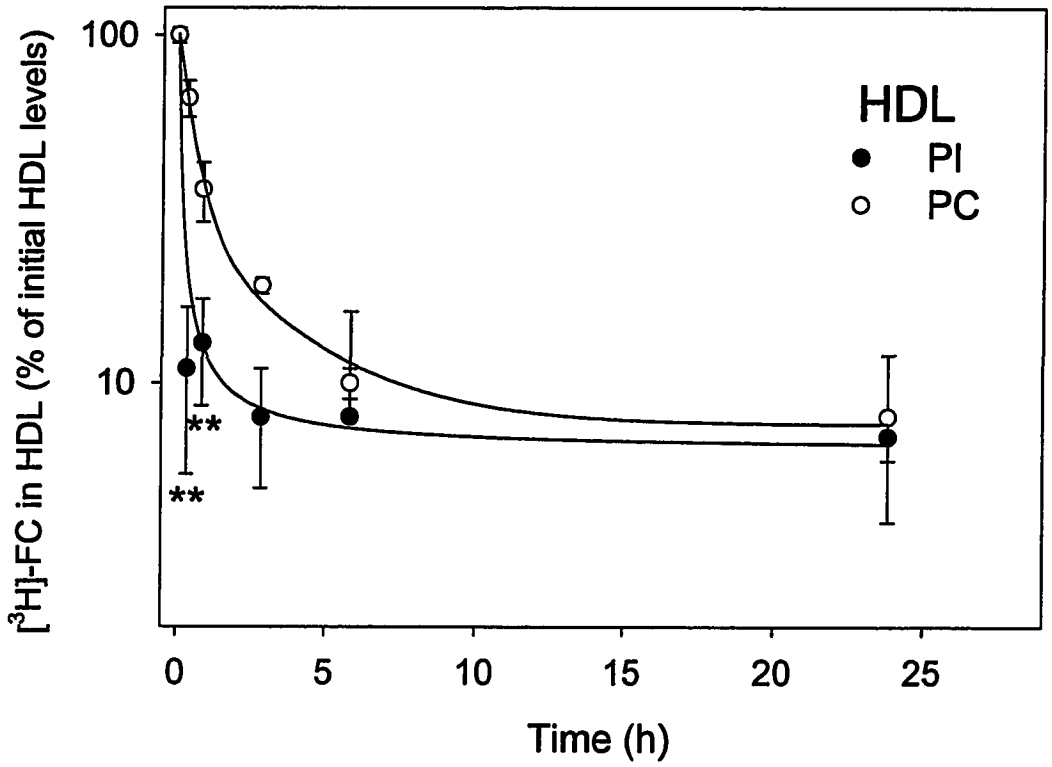
time point taken at 10 minutes post injection was defined as 100%. The clearance curves from the PI- and PC-injected animals were notably different in shape. The initial clearance rate of the PI-injected rabbit (as defined by the data points prior to the clearance-curve inflection point) was significantly greater than that of the PC-injected rabbit. Using the first 4 time points, initial [^{3}H]-tracer clearance rates (slope of curves) were calculated for each PI-injected rabbit (n = 3) and for each PC-injected rabbit (n = 3) and the means ± S.D. were determined as -78.3 ± 3.9 %/h and -50.6 ± 1.9 %/h, respectively. These values were determined to be significantly different (with $P < 0.001$) by the two-tailed Student's *t*-test for un-paired data. The conventional kinetic modelling approach does not fit the [^{3}H]-tracer clearance curve from the PI-injected rabbits and fractional catabolic rates could not be determined. This model, termed the two-pool model was defined by Le *et al*, and describes the existence of an irreversible and reversible clearance pool (102). The justifications for not using the two-pool model are described in the discussion. The base line levels of the two [^{3}H]-tracer clearance curves (PI vs. PC) were also different. The PI [^{3}H]-tracer clearance curve was cleared to a baseline level of ~20% while the PC curve continues to ~10% of the initial levels. The 24-hour time point in the PI-injected rabbit was significantly greater (~2-fold) than that of the PC-injected rabbit. Except for the 6- and 24-hour time point, the level of tracer was significantly less in the PI-injected rabbit than the PC-injected rabbit as determined by ANOVA and the post hoc Tukey Kramer Multiple Comparison Test (Figure 6). This also suggests that the [^{3}H]-cholesterol clearance rate is greater in the PI-injected rabbits than in the PC-injected rabbits.

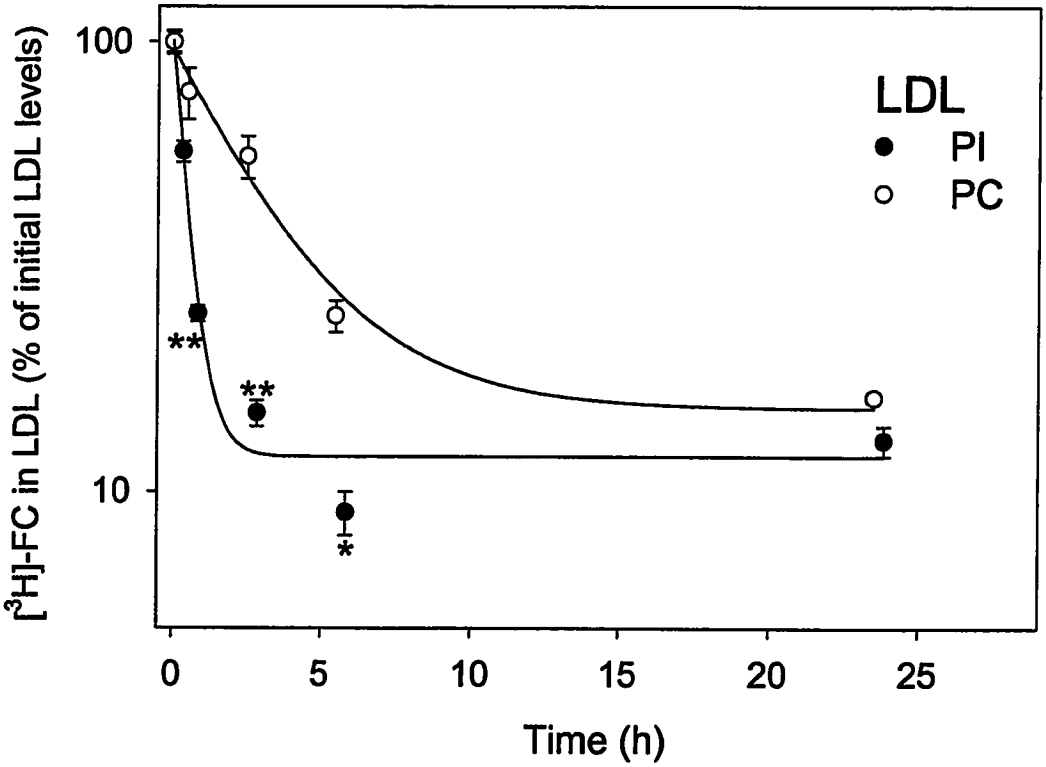
Figure 6. **The effects of PI-vesicle injections on the clearance of total [^{3}H]-radioactivity from the plasma in rabbits.** Rabbits were injected with PI- (36 mg) or PC- (31 mg) vesicles containing [^{3}H]-FC and plasma samples were taken. Radioactivity in the plasma was measured and the plasma clearance of total [^{3}H]-tracer was illustrated. Values are the means ± S.D. from three different PC rabbit injections or three different PI rabbit injections. The radioactivity in the plasma after 10 minutes post-injection was defined as 100%. A one-way ANOVA analysis determined there to be a high degree of significance of variance in the data $F(11,24) = 140$, $P < 0.001$. Post hoc analysis using the Tukey Kramer Multiple Comparison test was performed to compare the PI-injected rabbit tracer levels and the PC-injected rabbit tracer levels at each time point (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).


100
10
Total [3H]-radioactivity in plasma
(%of initial plasma levels)
PI
PC
***
***
**
*
0
5
10
15
20
25
Time (h)

The levels of [^{3}H]-FC (separated from CE by TLC) were measured in isolated lipoproteins. Figure 7 shows the clearance of [^{3}H]-FC from isolated HDL and LDL pools. The rate of clearance of HDL-FC and LDL-FC tracer appeared greater in PI-injected rabbits when compared to the PC-injected rabbits. This experiment with complete time-points was only performed in one PI- and one PC-injected rabbit and therefore represents preliminary data. Fifty percent of the HDL-FC was removed from the plasma within 12 minutes ($t_{1/2}$) in the PI-injected rabbit compared to ~40 minutes for the PC-injected rabbit. The LDL-FC clearance from the plasma was also accelerated ($t_{1/2}$ = 42 minutes) in the PI-injected rabbit when compared to ($t_{1/2}$ = 5 h) the PC injected rabbits. In general, the clearance of FC from HDL is much greater than in LDL in both rabbits. The total radioactivity associated with the lipoprotein pools were measured as a percentage of the total radioactivity in the PI- and PC-injected animals. The majority of the total plasma radioactivity at the initial 10-minute time point was recovered in the HDL (46 - 51%) and LDL (29 - 34%) pool. These ranges represent the values obtained from the PI- and PC-injected animals. Only a small percentage (<10%) of radioactivity was recovered in the VLDL fraction in the animals. This data appears to be in agreement with data presented in figure 6. This data suggests that the initial rapid clearance of tracer from the PI-injected rabbit (Figure 6) may be due to a rapid clearance of [^{3}H]-FC from the lipoprotein pool. This experiment must be repeated to validate this finding.

Figure 7. **The effects of PI-vesicle injections on the clearance of [^{3}H]-FC from HDL and LDL.** Rabbits were injected with PI- (36 mg) or PC- (31 mg) vesicles containing [^{3}H]-FC. Plasma samples were taken at various time points. Lipoproteins were isolated from plasma samples and the [^{3}H]-FC was separated from [^{3}H]-CE as described in the Experimental Procedures. The clearance of [^{3}H]-FC from HDL and LDL are illustrated. Values are the means ± S.D. of triplicate measurements from one PC-injected rabbit and from one PI-injected rabbits. The radioactivity ([^{3}H]-FC) in HDL or LDL 10 minutes post-injection is defined as 100%. A one-way ANOVA analysis determined there to be a high degree of significance of variance in the data for HDL ($F(11,24) = 164$, $P < 0.0001$) and LDL ($F(10,22) = 187$, $P < 0.0001$) from this single experiment. Post hoc analysis using the Tukey Kramer Multiple Comparison test was performed to compare the PI-injected rabbit [^{3}H]-FC levels and the PI-injected rabbit [^{3}H]-FC levels at each time point for HDL and LDL (* $P < 0.01$, ** $P < 0.001$).


100
10
HDL
PI
PC
**
**
0
5
10
15
20
25
Time (h)
[3H]-FC in HDL (% of initial HDL levels)


100
10
LDL
PI
PC
**
**
*
0
5
10
15
20
25
Time (h)
[3H]-FC in LDL (% of initial LDL levels)

The Effect of a PI-Injection on the Plasma Cholesterol Esterification Rates in a Rabbit

The effect of PC or PI injections on the rates of cholesterol esterification by LCAT was measured in individual plasma samples from the various time points. Only slight variations in LCAT activity were observed following the injection of PC-vesicles (n = 1) (Figure 8). In contrast, figure 8 shows that the initial endogenous LCAT activity was reduced by ~80%, 10 minutes following PI-injection (n = 1). PI-injection caused a reduction in the fractional rates of cholesterol esterification from 45% to 8% per hour. The LCAT activity returned to approximately 75% of pre-injection levels, 6 hours after the PI injection. The cholesterol esterification rates from the plasma of the PI injected animal at 10, 30 minutes, 1, 3 and 6 hour(s) were significantly lower than that of the PC injected control. This experiment was performed with only one control and one experimental rabbit and must be repeated.

The Effect of PI on the Plasma Cholesterol Esterification Rate in vitro

Studies were performed to confirm that PI could inhibit plasma LCAT activity *in vitro*. Human plasma was obtained from a male normal lipidemic fasting volunteer. Rabbit plasma was obtained from a fasting rabbit. Human and rabbit plasma were enriched with 1 mg/ml of PI-vesicles, PC-vesicles or PBS and fractional esterification activity was measured. Figure 9 indicates that rabbit plasma has a higher rate of esterification (approximately 3-fold) than human plasma. The addition of PC-vesicles to rabbit plasma appeared to have negligible effects on the endogenous LCAT activity when compared to control rabbit plasma. Figure 9 shows there was an increased rate of esterification (~50%) in PC-enriched human plasma compared to control human plasma.

Figure 8. **The effects of PI-injection in rabbits on the cholesterol esterification by LCAT.** Rabbits were injected with PI- (36 mg) or PC- (31 mg) vesicles and then plasma samples were drawn at various time points. Plasma samples were incubated in the presence of [^{3}H]-FC and the rates of formation of CE by LCAT were measured as described in the Experimental Procedures. The fractional esterification rate was calculated as the percentage of FC converted to CE per hour and expressed as a percent of the pre-injection plasma sample value. Values are the mean ± S.D. of triplicate determination from one PI-injected rabbit and from one PC-injected rabbit. A one-way ANOVA determined there to be a high degree of significance of variance in the data from this single experiment $F(11,24) = 84.1$, $P < 0.0001$. Post hoc analysis using the Tukey Kramer Multiple Comparison test was performed and compared PI-injected rabbit values to the PC-injected rabbit values (* $P < 0.001$).

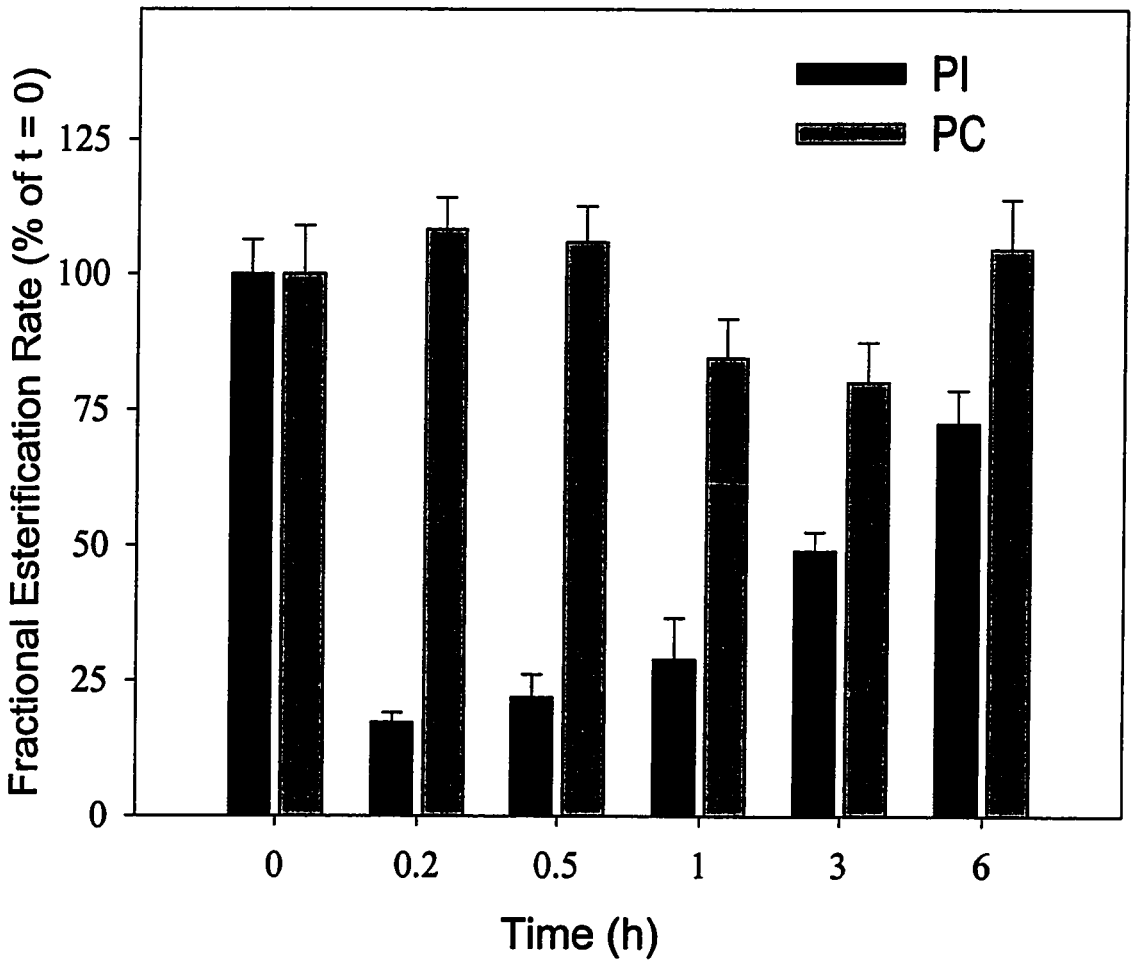
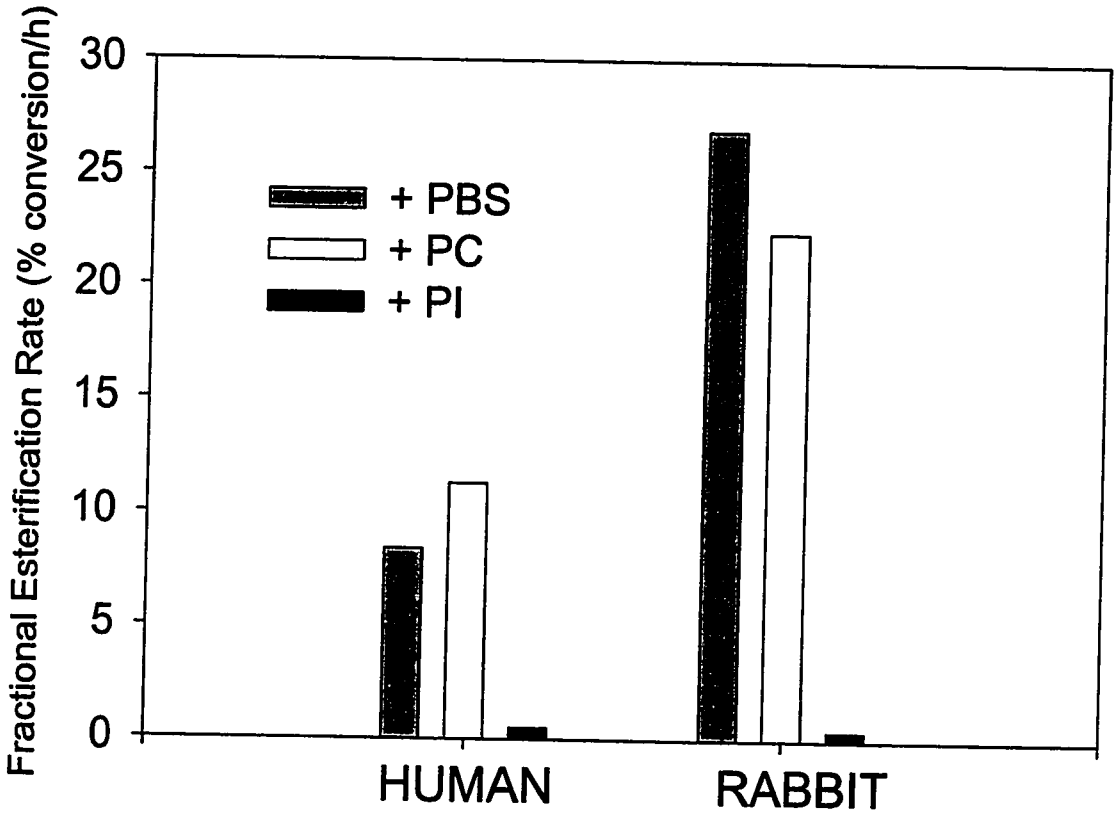

PI
PC
Fractional Esterification Rate (% of t = 0)
125
100
75
50
25
0
0
0.2
0.5
1
3
6
Time (h)

Figure 9. **The effects of PI added to human and rabbit plasma on the cholesterol esterification by LCAT *in vitro*.** Fasting rabbit or human (normal lipidemic male) plasma was incubated with 1 mg/ml of PI- or PC-vesicles or PBS at 4°C for 48 hours. Fractional esterification assay was performed as described in the Experimental Procedures. The fractional esterification rate is expressed as the percentage of FC converted to CE per hour. Numbers represented in parentheses indicates the number of times the experiments was performed. When n = 2 the values represent the average (range) from 2 separate experiments. When n = 3 values represent the mean ± S.D. from 3 separate experiments. Significance of difference was determined using the un-paired two-tailed Student's *t*–test to compare the PI human plasma value to the PBS human plasma value (* $P < 0.001$).


Fractional Esterification Rate (% conversion/h)
30
25
20
15
10
5
0
+ PBS
+ PC
+ PI
HUMAN
RABBIT

The addition of 1 mg/ml of PI-vesicles to plasma inhibited the fractional esterification rate of endogenous LCAT to background levels in both rabbit and human plasma, which was in agreement with the data obtained from the PI-injected rabbit. Although, no significance of difference could be determined from the values obtained in experiments that were only repeated twice, it could be suggested that the addition of PI inhibited the rabbit plasma esterification rate *in vitro*. However, human plasma incubated with PI (n = 3) had significantly lower esterification rates when compared to human plasma incubated with equivalent volumes of PBS (n = 3). This indicated that the addition of PI-vesicles could significantly inhibit human plasma esterification rates.

The Effects of Injections of PI-vesicles or PI-HDL on [^{3}H]-FC in the Liver and Bile

To evaluate the involvement of the liver in plasma cholesterol clearance, preliminary studies examined hepatic uptake and biliary secretion of [^{3}H]-FC in PI- (n = 1) and PC-injected animals (n = 1). Table VI shows a ~50% increase in total liver [^{3}H]-FC in the PI-injected rabbits relative to PC-injected rabbits, 30 minutes following injection. The total secretion of [^{3}H]-FC into the bile in the PI-injected rabbits was also about 20-fold greater than the biliary levels in PC-injected rabbits after 30 minutes. Both rabbits were injected with ~6.5 x 10^8 cpm.

To examine the involvement of HDL in hepatic cholesterol uptake, PI- or PC-enriched (and [^{3}H]-FC labelled) HDL was injected into rabbits and liver and biliary [^{3}H]-FC was measured. Rabbits injected with PI-enriched HDL had 3.6-fold more [^{3}H]-FC secreted into the bile after 30 minutes, but had similar levels of radioactivity in the liver when compared to control rabbits (Table VI). Each rabbit was injected with 2 mg of either PI- or PC-enriched HDL and ~1.7x 10^8 cpm.

Table VI. The effects of an injection of PI-vesicles or PI-enriched-HDL on cholesterol transport to the liver and secretion in the bile in rabbits. †

Injectate	Total Bile Radioactivity[a] (cpm x 10^{-3})	Total Liver Radioactivity[b] (cpm x 10^{-6})
PI-vesicles (n = 1)	*104.3 ± 2.9	*164 ± 9
PC-vesicles (n = 1)	4.7 ± 0.4	101 ± 7
PI-HDL (n = 1)	*16.1 ± 0.2	21 ± 2
PC-HDL (n = 1)	4.4 ± 0.7	25 ± 2

†Rabbits were injected with PI- (36 mg) or PC- (31 mg) vesicles or PI- or PC-enriched HDL (2 mg) containing [^{3}H]-FC. Rabbits were euthanised 30-minutes post-injection. Liver and gall bladder were removed. The quantity of [^{3}H] in the bile and liver were measured as described in the methods. Significance of difference was calculated using the un-paired two-tailed Students t-test and indicated the value was significantly different from the respective PC control (* $P < 0.001$).

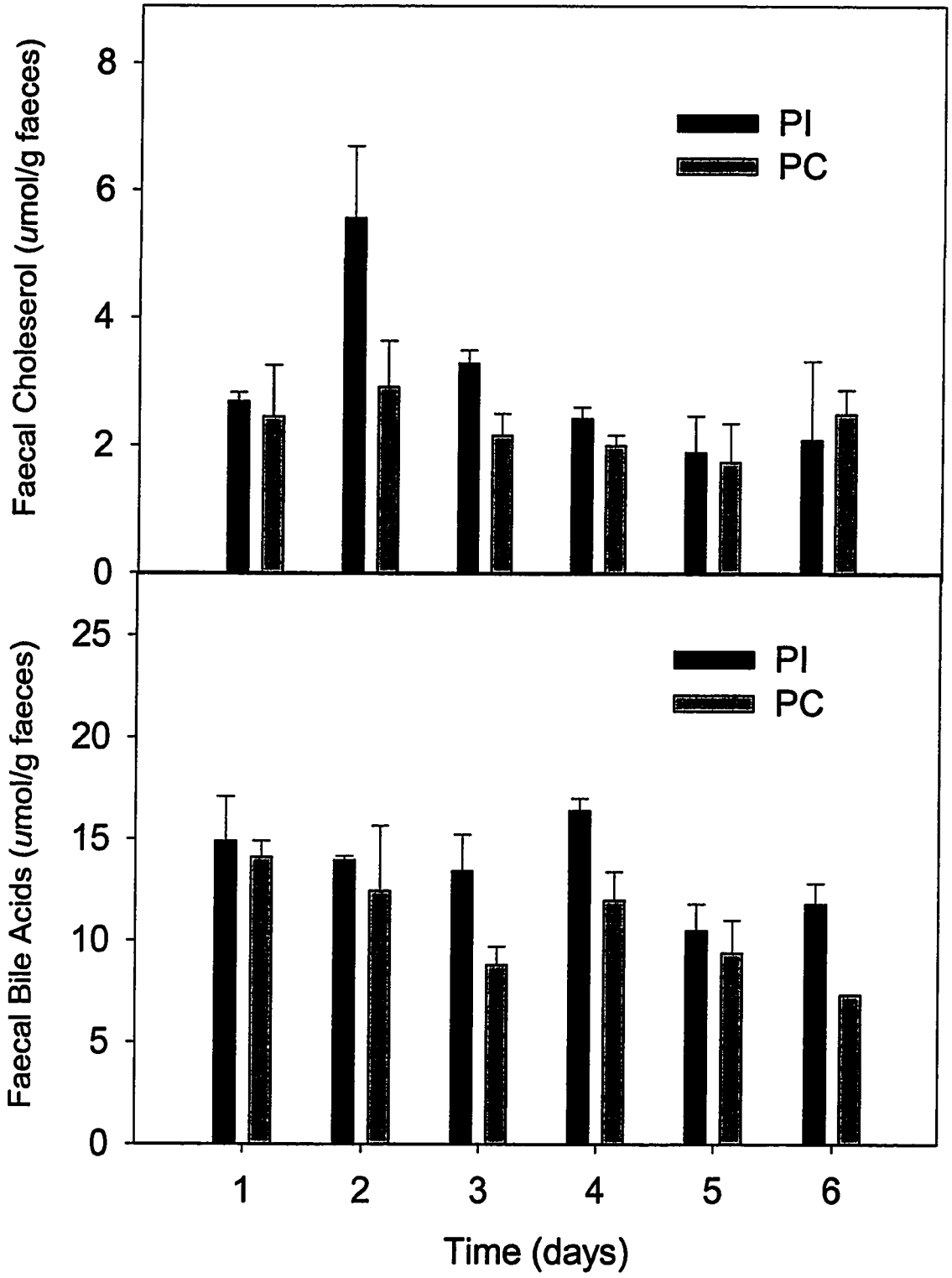
[a] Each value represents the mean ± S.D. of triplicate measurements of bile sample from one rabbit.

[b] Each value represents the mean ± S.D. for 3 independent tissue extractions from one rabbit liver.

The Effects of PI-Injections on Cholesterol and Bile Acid Levels in the Faeces

Faecal samples were analysed to determine if sterol output increased following the bolus injection of phospholipids vesicles. Figure 10 compares the bile acid and sterol faecal concentration in the rabbits injected with PI (36 mg) (n = 2) vesicles with rabbits injected with PC (31 mg) (n = 2) vesicles. The data (Figure 10) show the average of the two means from the two PI-injected rabbits and the two PC-injected rabbits. The total bile acids in the faeces fluctuated between 7 and 16 μmol/g in both the PI- and PC-injected rabbits analysed over six days post-injection. Total cholesterol faecal levels in the two PC-injected rabbits ranged from 1.1 μmol/g to 3.6 μmol/g over the six-day period. The total cholesterol concentration in the faeces of the PI-injected rabbits increased in both rabbits from day one to day two by ~2-fold (70% and 140% increase). This peak on day two was followed by a return to day 1 levels by day 3 and 4.

Figure 10. **The effects of an injection of PI on total cholesterol and total bile acid levels in the faeces.** Rabbits were injected with PI- (36 mg) (n = 2) or PC- (31 mg) (n = 2) vesicles and faeces were collected for 6 days. Lipids and bile acids were extracted from each daily sample and measured as described in the Experimental Procedures. Each daily determination represents the average (range) of the daily mean values obtained from two separate PI-injected rabbits and two separate PC-injected rabbits. Each daily mean value determined from each rabbit was a result of a triplicate faecal extraction and measurement.


Faecal Cholesterol (umol/g faeces)
8
6
4
2
0
PI
PC
Faecal Bile Acids (umol/g faeces)
25
20
15
10
5
0
PI
PC
1
2
3
4
5
6
Time (days)

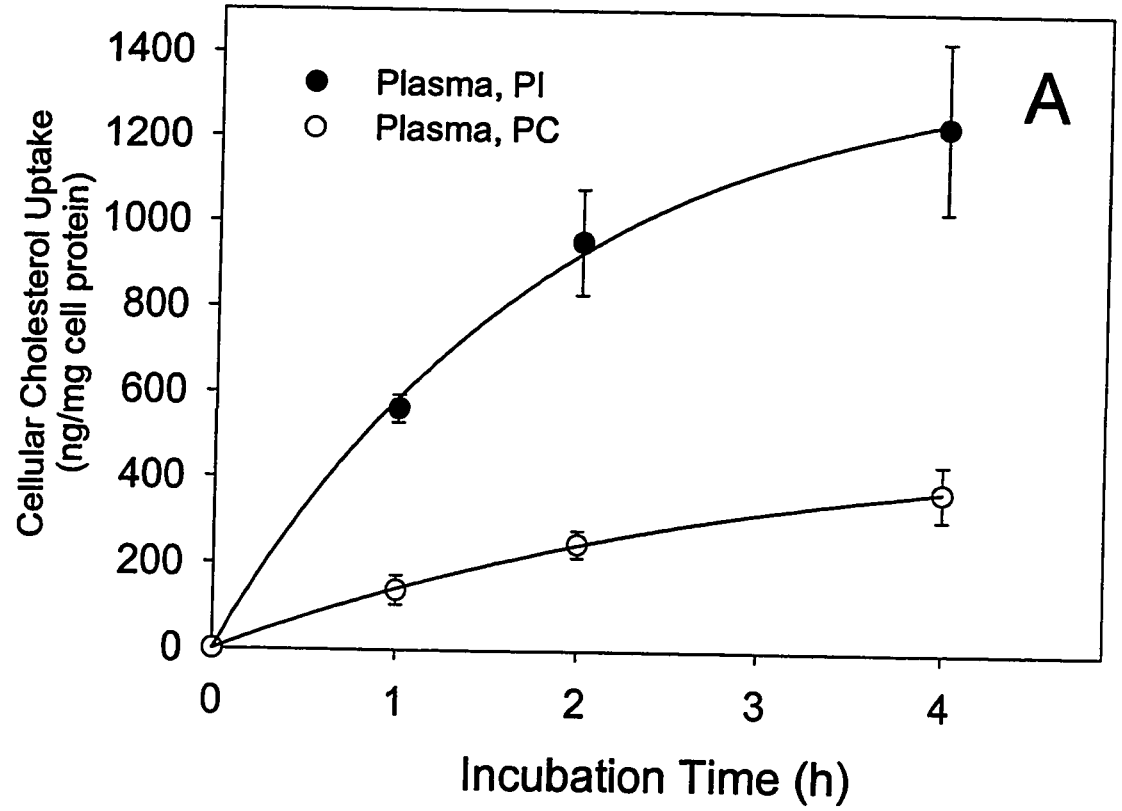
Total faecal dry-mass output in all rabbits remained relatively constant over the 6 days of sampling (50-70 grams/day).

The Effects of PI on Cellular Cholesterol Uptake in HepG2 Cells

To explore the role of the hepatocyte in the PI-enhanced cholesterol clearance, cellular [^{3}H]-FC uptake from PI- or PC-enriched plasma was examined in cultured liver cells. Human hepatoma liver cells, HepG2, were incubated with medium containing 5% (v/v) human plasma that had been enriched with either PI- or PC-vesicles. Human plasma was obtained from fasting normal lipidemic male volunteers. Figure 11A shows a cellular FC-uptake time-course from plasma enriched with either PI- or PC- vesicles obtained from a single experiment. This experiment suggests that PI-enriched plasma stimulates FC-uptake when compared to PC-enriched plasma. Figure 11B confirms that plasma enriched with PI-vesicles (n = 4) mediates a significantly greater increase in cellular cholesterol uptake (~2-fold), as compared to PC-enriched plasma (n = 4) preparations after incubation with cells for one hour at 37°C. Although the standard deviation appears relatively large in figure 11B, values were determined to be significantly different using the unpaired two-tailed Student's *t*-test ($P < 0.05$). PI-enrichment of plasma did not appear to enhance CE-uptake in HepG2 cells as suggested from studies with [^{3}H]-CE labelled plasma (Figure 12). CE uptake culture studies would have to be repeated to confirm that PI specifically enhances FC uptake.

The most probable plasma constituents to be involved in the flux of cholesterol to the cells are the HDL and LDL class of lipoproteins. Therefore, cellular uptake of cholesterol was evaluated from human HDL or LDL that had been isolated and enriched with PI or PC.

Figure 11. **The effects of PI on the cellular uptake of plasma-FC by HepG2 cells.** HepG2 cells were grown as described in the Experimental Procedure and were incubated at 37°C for 1, 2 or 4 hours or with EMEM containing 2 mg/ml BSA and 5% fasting human plasma, which had been previously enriched with either PI or PC and labelled with [^{3}H]-FC. Cells were washed and the associated radioactivity was measured as described in the Experimental Procedure. Cholesterol mass uptake was calculated using plasma-FC specific activity values. (Panel A) Values represent the mean ± S.D. for triplicate determinations (n = 3 wells) from an experiment performed once. (Panel B) Values represent the mean ± S.D. from four separate experiments. Significance of difference was calculated using the un-paired two-tailed Students t-test (* $P < 0.05$).


Plasma, PI
Plasma, PC
A
Cellular Cholesterol Uptake
(ng/mg cell protein)
1400
1200
1000
800
600
400
200
0
0
1
2
3
4
Incubation Time (h)

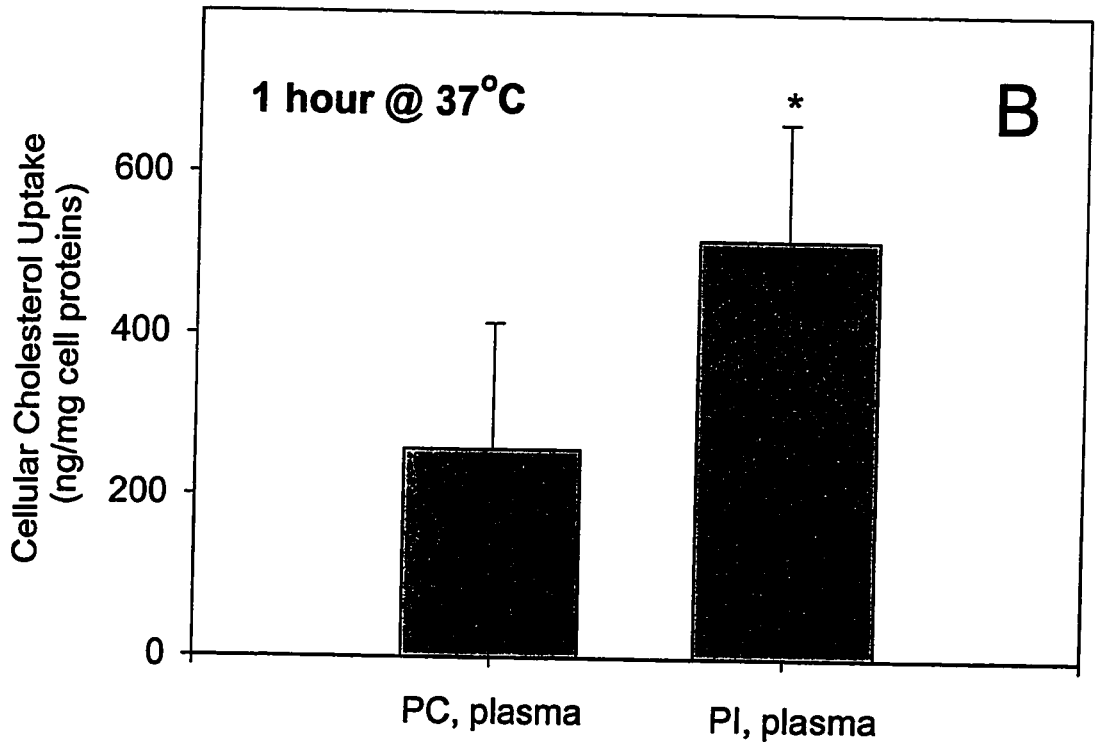
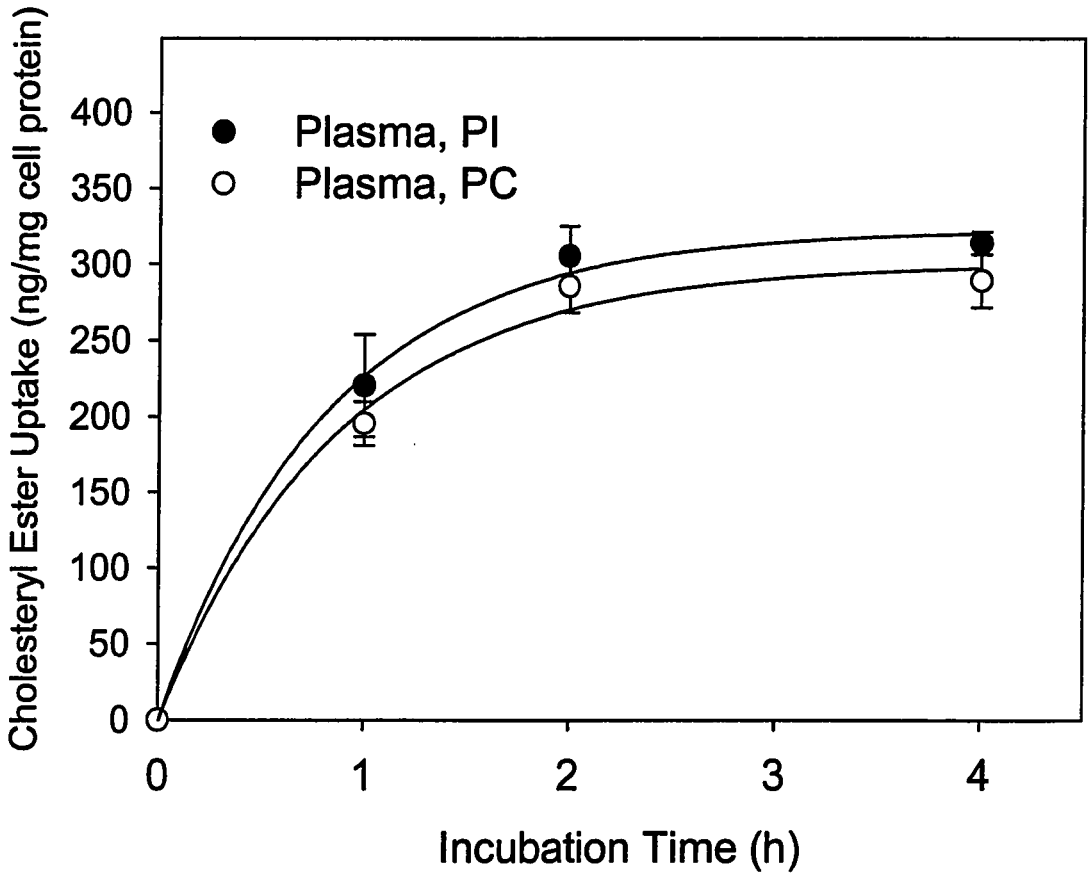

1 hour @ 37°C
B
*
Cellular Cholesterol Uptake
(ng/mg cell proteins)
600
400
200
0
PC, plasma
PI, plasma

Figure 12. **The effects of PI on the cellular uptake of plasma-CE by HepG2 cells.** HepG2 cell monolayers were grown as described in the Experimental Procedure and were incubated at 37°C for 1, 2 or 4 hours with EMEM containing 2 mg/ml BSA and 5% plasma, which had been previously enriched with either PI or PC and labelled with [^{3}H]-CE. Cells were washed and the associated radioactivity was measured as described in the Experimental Procedures. Cholesteryl ester mass uptake was calculated using plasma-CE specific activity values. Values represent the mean ± S.D. for triplicate determinations (n = 3 wells) from an experiment performed once.


Cholesteryl Ester Uptake (ng/mg cell protein)
400
350
300
250
200
150
100
50
0
Plasma, PI
Plasma, PC
0
1
2
3
4
Incubation Time (h)

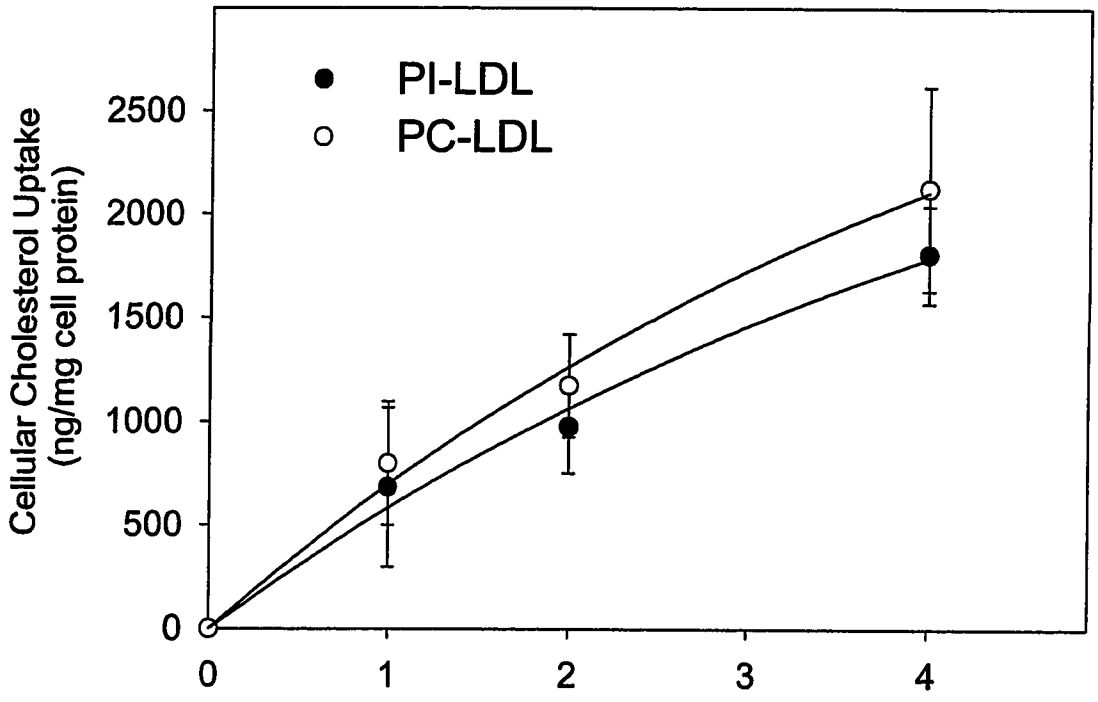
Cells that were incubated with medium containing 50 μg/ml of PI-enriched HDL (n = 6) showed a statistically significant increase in cellular uptake of cholesterol, when compared to PC-enriched HDL (n = 6) after 2 and 4 hours (Figure 13). Untreated HDL (n = 2) experiments indicated that the FC uptake was similar to that of the PC-enriched HDL. PI-enriched LDL cholesterol uptake (n = 3) showed similar FC uptake values when compared to the control PC-enriched LDL (n = 3) (Figure 13).

Studies performed to evaluate HDL-protein cell association indicated that the PI-enriched HDL cell association was approximately ~20% greater when compared to the PC-enriched HDL uptake (Figure 14). ANOVA and subsequent analysis using the Tukey Kramer Multiple comparison test indicated that at 2 and 4 hours, the PI-HDL cell association was significantly greater than that of the PC-enriched HDL. This experiment was only performed once and therefore would have to be repeated so that conclusions could be made on the effect of PI on HDL cell association.

Cellular FC-Uptake in ldlA7 and ldlA7-SR-BI Cell lines

In order to evaluate the role of the SR-BI in PI-enhanced cholesterol uptake mechanism from HDL; cell culture studies were performed using a CHO cell lines that expressed low levels of the SR-BI (ldlA7) and another cell line that over-expressed the receptor (ldlA7-SR-BI). This cell line lacks a functional LDL receptor and therefore allows for a more controlled analysis of the effect of SR-BI on FC-HDL uptake. Figure 15 shows that the ldlA7-SR-BI cells have a ~four-fold higher rate of cellular cholesterol uptake from HDL when compared to the ldlA7 cells, which is in agreement with reported values in the literature (197). However, there was no obvious effect of PI-enrichment on cholesterol uptake from HDL in either the ldlA7 or the ldlA7-SR-BI cells (Figure 15).

Figure 13. **The effects of PI on the cellular uptake of LDL- and HDL-FC by HepG2 cells.** HepG2 cell monolayers were grown as described in the Experimental Procedures and were incubated at 37°C for 1, 2 or 4 hours with EMEM containing 2 mg/ml BSA and LDL (50 μg/ml) (Top Panel) or HDL (50 μg/ml) (Bottom Panel), which was enriched with PI or PC and labelled with [^{3}H]-FC. Cell associated radioactivity was measured. Cholesterol mass uptake was calculated using HDL- or LDL-FC specific activity values. PC-LDL (n = 3) and PI-LDL (n = 3) values represent the mean ± S.D. from 3 separate cell experiments. PC-HDL (n = 6) and PI-HDL (n = 6) values represent the mean ± S.D. from six separate cell experiments. HDL values represent the average (range) from two separate cell experiments. A one-way ANOVA was performed and revealed that variations between PI-HDL and PC-HDL groups were statistically significant, $F(5, 30) = 49.6$, $P < 0.0001$. Post hoc analysis was performed using the Tukey Kramer Multiple Comparison Test (* $P < 0.001$)


PI-LDL
PC-LDL
Cellular Cholesterol Uptake
(ng/mg cell protein)
2500
2000
1500
1000
500
0
0
1
2
3
4

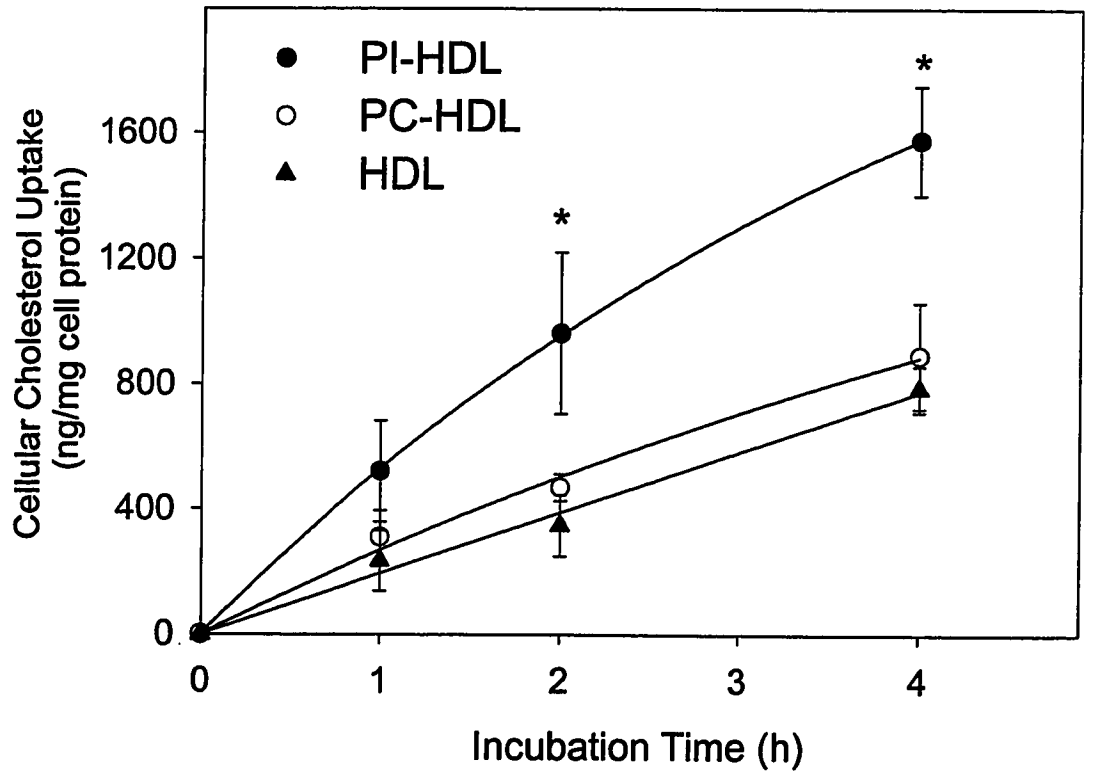

PI-HDL
PC-HDL
HDL
*
*
Cellular Cholesterol Uptake
(ng/mg cell protein)
1600
1200
800
400
0
0
1
2
3
4
Incubation Time (h)

Figure 14. **The effects of PI on cellular association of HDL-protein by HepG2 cells.** HepG2 cell monolayers were grown as described in the Experimental Procedure and were incubated at 37°C for 1, 2 or 4 hours with EMEM containing 2 mg/ml BSA and ^{125}I-HDL (50 μg/ml) that was enriched with PI or PC. Cell associated radioactivity was measured as described in the Experimental Procedure. HDL-protein mass uptake was calculated using HDL-protein specific activity values. Values represent the mean ± S.D. for triplicate determinations (n = 3 wells) from an experiment performed once. One-way ANOVA was performed and revealed that variations between PI-HDL and PC-HDL in this single experiment were statistically significant, $F(5, 30) = 49.6$, $P < 0.0001$. Post hoc analysis was performed using the Tukey Kramer Multiple Comparison Test (* $P < 0.05$).

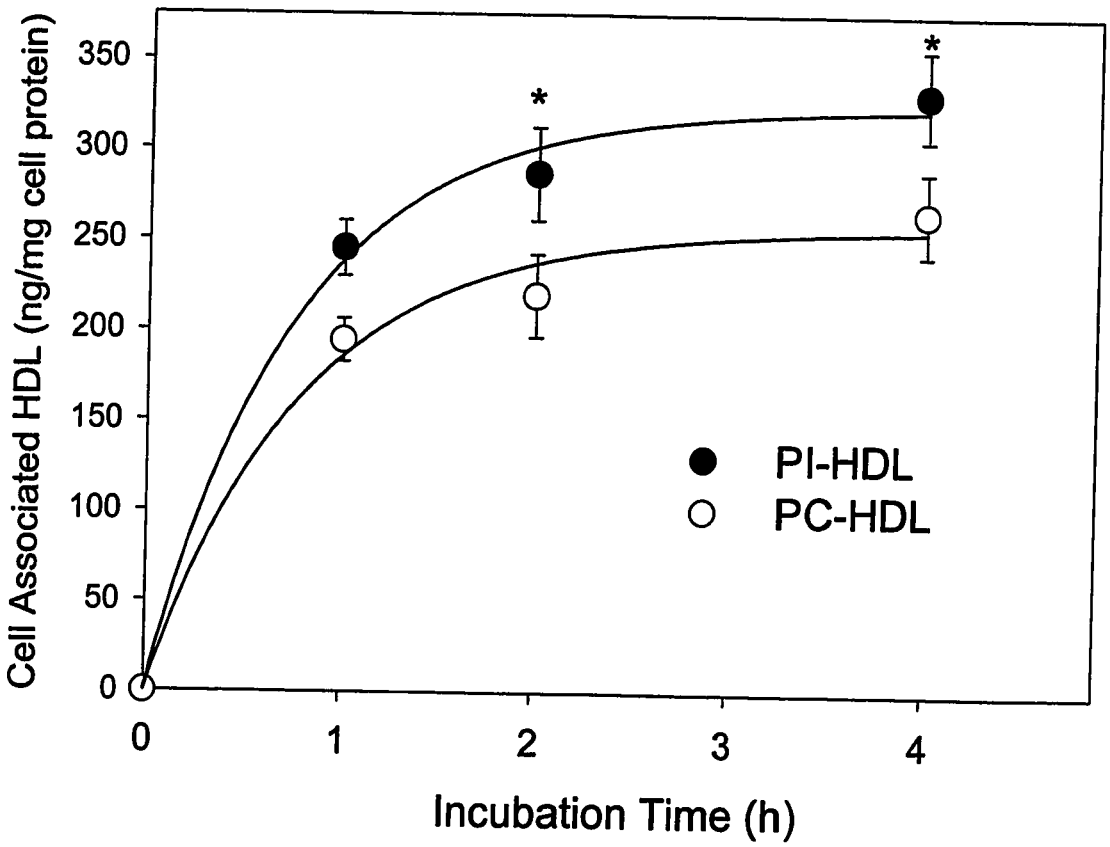
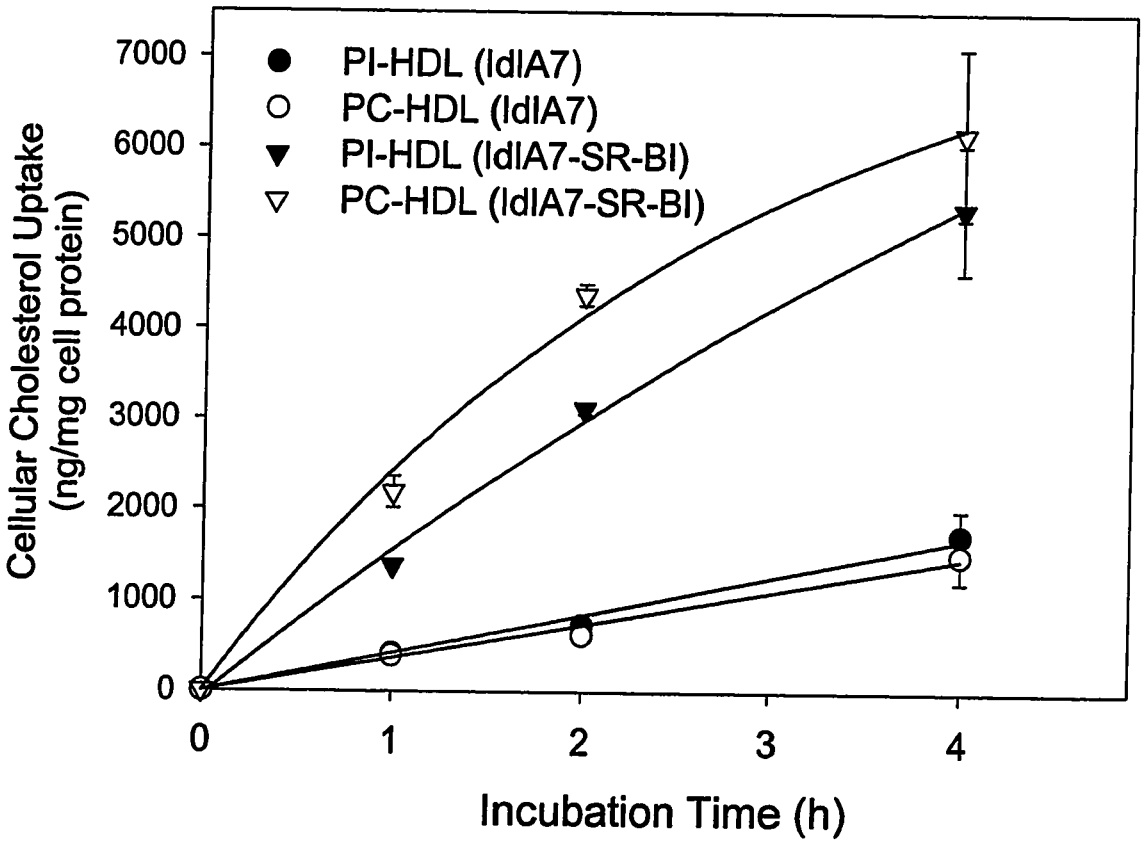

Cell Associated HDL (ng/mg cell protein)
350
300
250
200
150
100
50
0
0
1
2
3
4
Incubation Time (h)
*
*
PI-HDL
PC-HDL

Figure 15. **The effects of PI on cellular uptake of HDL-FC in ldlA7 and ldlA7-SR-BI cells.** ldlA7 and ldlA7-SR-BI cell monolayers were grown as described in the Experimental Procedure. Cells were incubated at 37°C for 1, 2 or 4 hours with DMEM containing 2 mg/ml BSA and HDL (50 μg/ml), that has been enriched with either PI or PC and labelled with [^{3}H]-FC. Cell associated radioactivity was measured as described in the Experimental Procedures. Cholesterol mass uptake was calculated using HDL-FC specific activity values. Values represent the average (range) from two independent experiments.


PI-HDL (IdlA7)
PC-HDL (IdlA7)
PI-HDL (IdlA7-SR-BI)
PC-HDL (IdlA7-SR-BI)
Cellular Cholesterol Uptake
(ng/mg cell protein)
7000
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
Incubation Time (h)

The Modulation of PI-Enhanced FC-Uptake in HepG2 Cells

To address the possibility that an ATP-binding cassette transporter, the MDR1 gene encoding a p-Glycoprotein, is involved in the uptake of cholesterol, inhibitor studies were performed. MDR1 is highly expressed in hepatocytes and has been shown to be expressed specifically in the HepG2 cell line (83). Evidence suggests that it may play a role in the uptake of cholesterol in intestinal cells (28). In this study, the cell modulators chelerythrine chloride (a PKC inhibitor) and propranolol (phosphatidic acid phosphohydrolase (PAPH) inhibitor) were used as they have previously been reported to inhibit cellular MDR1 function (201; 202; 203). Although, these inhibitors are not specific for MDR1, they provide a starting point for the analysis of the mechanism involved in FC uptake from PI-enriched HDL. To determine the involvement of PKC in the PI-stimulated uptake of cholesterol, HepG2 cells were pre-treated with a PKC inhibitor, chelerythrine chloride, and cholesterol uptake studies from PI-enriched HDL or PC-enriched HDL were performed. Figure 16 shows that the addition of 10 μM of chelerythrine chloride to the cells inhibited the cholesterol uptake from PI-enriched HDL by ~50%. The cholesterol uptake from the PC-enriched HDL was also inhibited (~25% of PC-HDL value) by the inhibition of PKC. Treatment with PMA (40 μM), a PKC activator (204), appeared to have had no effect on cholesterol uptake from HDL (Figure 17).

Cellular cholesterol uptake was also inhibited in cells that were treated with 200 μM propranolol. Figure 16 shows that cholesterol uptake from both PI- and PC-enriched HDL is inhibited by ~50% when treated with propranolol. Both inhibitors used in this study appeared to inhibit cellular uptake of FC from HDL, yet no conclusions can be

made on the role of MDR1 in this process. Additional techniques that are more specific would have to be used to implicate this transporter in the cellular FC uptake pathway.

To address the possibility that PI may be acting independently of HDL, cells were pre-treated with medium in the presence or absence of PI-vesicles (10 μg/ml = 11 μM) prior to the incubation with medium containing [^{3}H]-FC labelled HDL. No difference in HDL cholesterol-uptake was observed between PI-pre-treated and untreated cells after 4 hours at 37°C (Figure 17).

Figure 16. **The effects of PI on cellular uptake of HDL-FC and inhibition with chelerythrine and propranolol.** The cell monolayers were grown as described in the Experimental Procedures. Cells were incubated at 37°C for 1 hour with EMEM containing 2 mg/ml BSA with/without 10 μM chelerythrine chloride (+ CC) or 200 μM of propranolol (+ Prop.). Medium was removed and the cell monolayer was then incubated at 37°C for 4 hours with EMEM containing 2 mg/ml BSA and HDL (50 μg/ml) that was enriched with PI or PC and labelled with [^{3}H]-FC. Cell-associated radioactivity was measured as described in the Experimental Procedures. Cholesterol mass uptake was calculated using HDL-FC specific activity values. All values from each experiment were expressed as a percent of the PC-HDL values (100%). Values represent the average (range) from two independent cell experiments.

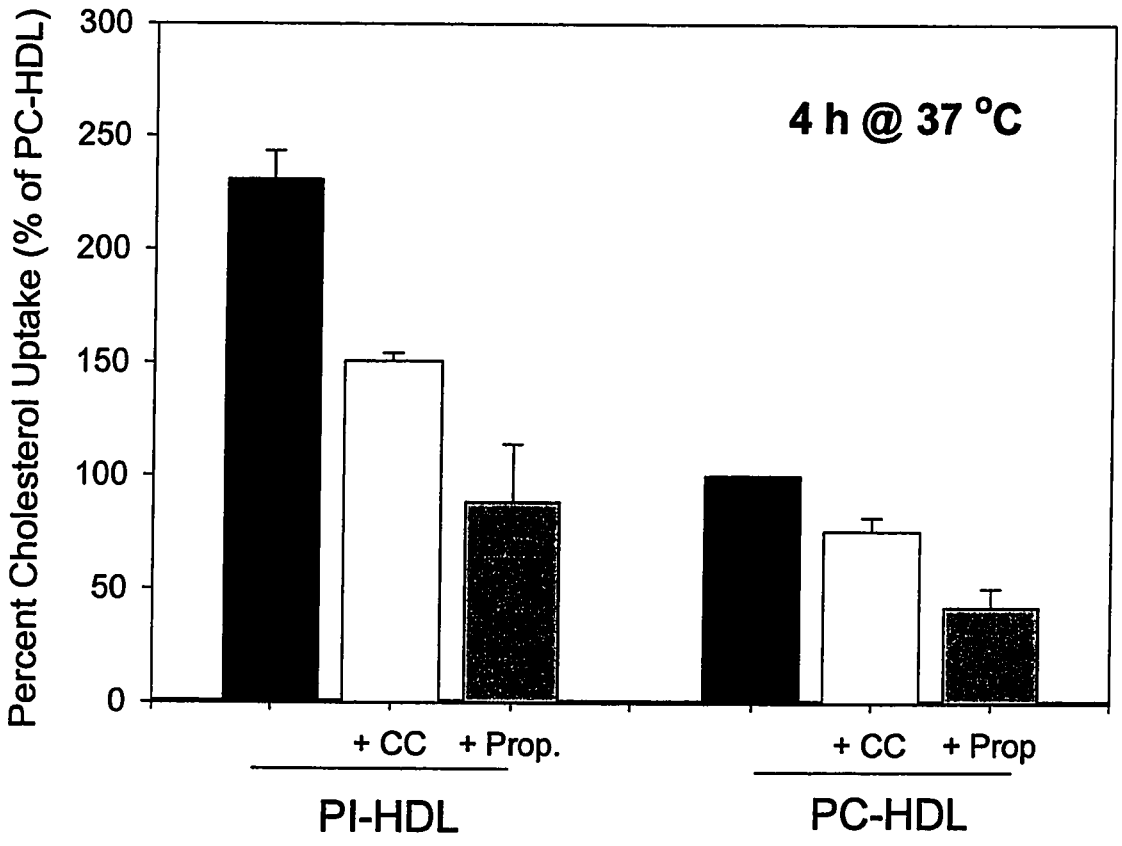
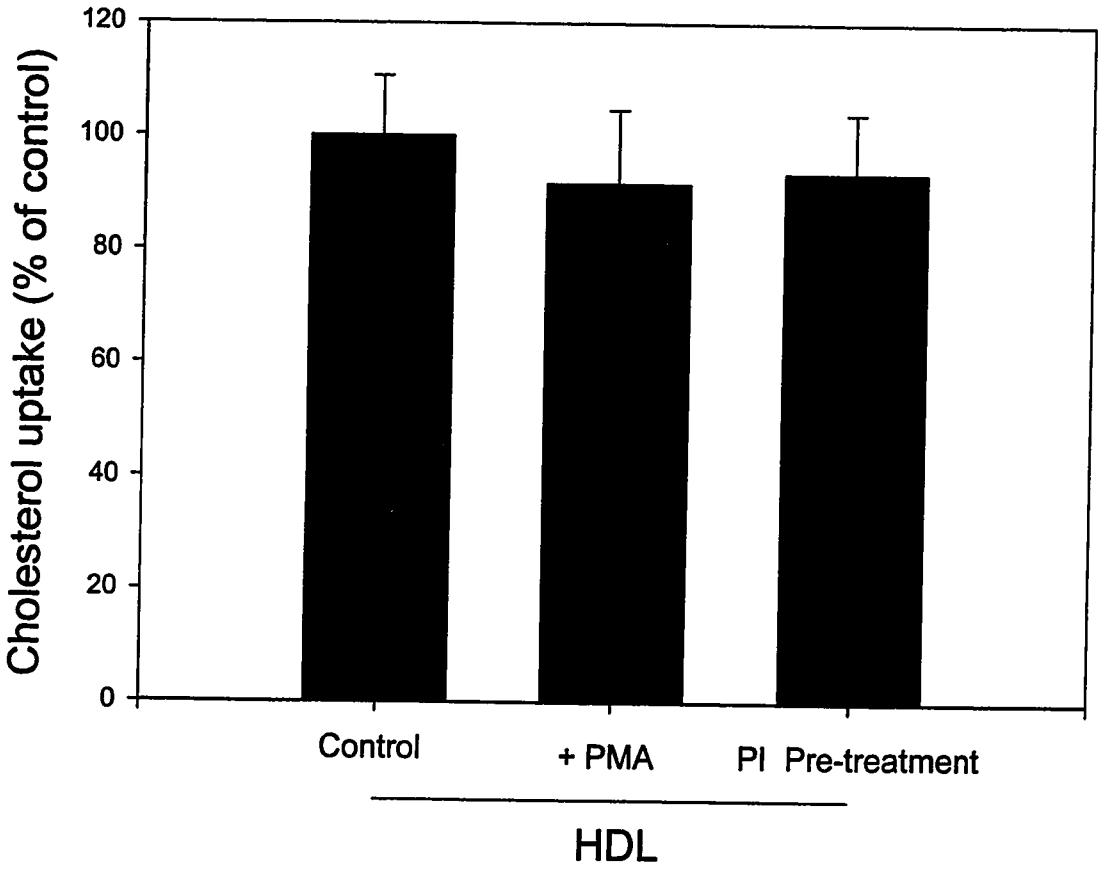

Percent Cholesterol Uptake (% of PC-HDL)
300
250
200
150
100
50
0
4 h @ 37 °C
+ CC
+ Prop.
+ CC
+ Prop
PI-HDL
PC-HDL

Figure 17. **The effects of PMA- and PI-pre-treatment on cellular uptake of HDL-FC in HepG2 cells.** Cell monolayers were grown as described in the Experimental Procedures. Cells were incubated at 37°C for 1 hour with EMEM containing 2 mg/ml BSA with/without 40 μM PMA or 11 μM PI-vesicles. Medium was removed and the cell monolayer was then incubated at 37°C for 4 hours with EMEM containing 2 mg/ml BSA and HDL (50 μg/ml) that was labelled with [^{3}H]-FC. Cell-associated radioactivity was measured as described in the Experimental Procedures. All values from each experiment are expressed as a percent of the HDL control value (100%). Values represent the mean ± S.D. for triplicate determinations (n = 3 wells) from one experiment.


Cholesterol uptake (% of control)
120
100
80
60
40
20
0
Control
+ PMA
PI Pre-treatment
HDL

DISCUSSION

Studies have shown that alterations in the composition of the lipoprotein particles can affect their charge and structural properties. These lipoprotein properties influence lipoprotein remodelling and plasma lipid metabolism (205-208). The major factor that alters lipoprotein surface charge is the content of charged molecules, such as proteins and anionic phospholipids (55). The enzymes and proteins involved in lipoprotein metabolism must interact through the surface lipid interface. A change in the lipoprotein surface charge alters the enzyme/lipoprotein interactions and thereby affects the metabolism of the lipoprotein. *In vitro* experiments have shown that the alterations in lipoprotein charge affects many of the enzymes involved in lipid and lipoprotein metabolism (45; 52; 124; 209; 210). The objective of this thesis is to evaluate the effects of an alteration in lipoprotein surface charge on cholesterol metabolism *in vivo*.

Lipoprotein charge was altered through the enrichment of the lipoprotein surface layer with anionic phospholipids and evaluated by agarose gel electrophoresis. *In vitro* experiments performed in this laboratory have shown that an increase in lipoprotein negative surface charge can be observed after incubation of individual lipoproteins, or plasma with anionic lipid vesicles. This suggests that these lipid molecules are absorbed into the surface layer of the lipoprotein. The lipid absorption in lipoproteins has been observed following incubations with PI- and PS-vesicles. Lipid-vesicles provide us with a useful tool that allows the controlled manipulation of lipoprotein charge both *in vitro* and *in vivo*.

To evaluate the effects of anionic lipids on cholesterol metabolism *in vivo*, rabbits were injected with 36 mg of PI-vesicles or 31 mg of PC-vesicles. Although both PI and PS vesicles were shown to alter lipoprotein charge *in vitro*, PI-vesicles were chosen for the rabbit studies for several reasons. PI is the most abundant anionic phospholipid in the plasma and in HDL (55) and therefore alterations in the concentration may be more physiologically relevant. The injection of PS-vesicles was considered more complicated because of the role that PS plays in the triggering of the blood coagulation cascade. Therefore, initial studies focused on the effects of PI on lipoprotein surface charge. These results show that injections of PI-vesicles into rabbits caused an increase in the negative surface charge of all classes of lipoproteins (Figure 5). This change in lipoprotein charge was directly due to the incorporation of the anionic lipids into the lipoproteins. The negative charge of the HDL pool reached maximal charge after 10 minutes post-injection while LDL and VLDL peaked later (1 hour and 30 minutes, respectively) and had less change in surface charge (Figure 5). The TLC method used to measure the PI levels in the lipoproteins was not sensitive enough to evaluate background levels or some post PI injected levels. The sensitivity of the technique would have to be improved or alternatively a different technique should be used in order to characterize the specific changes in the lipoprotein PI levels following the vesicle injection. The PI mass measurements obtained suggested that the PI-vesicles associated with HDL and that the PI composition increased from being below detectable levels to approximately 10% of the total HDL phospholipids after 10 minutes. These results are in agreement with the earlier studies by Nilsson *et al* who examined the fate of an intravenous injection of PI in rabbits and reported it was rapidly transferred to the HDL molecules where the majority

of the lipid resides in the blood (60). The preferential absorption of PI into HDL may be due to the proportionally higher levels of HDL in the plasma in the rabbit model. This group also reported that the clearance of PI was slower than the clearance of PC, possibly because PI was hydrolysed more slowly by HL and LPL than other phospholipids (60). The clearance of PI from the different lipoproteins may have been due to a lipoprotein-cellular transfer or to a selective hydrolysis by a phospholipid lipase.

The clearance of [^{3}H]-tracer (FC and CE) from plasma was monitored and the data showed an increase in the clearance rate from plasma of the PI-injected rabbits when compared to the PC-injected control rabbits. The shapes of the clearance curves were remarkably different. The tracer levels in the PI-injected animals were cleared almost immediately to a level that was ~20% of the initial injected levels, while the PC-injected animal were cleared to ~10% of the initial levels much later. Normally a two-pool model can be used to determine the fractional catabolic rates of metabolites being cleared from the plasma. This model assumes the existence of an intravascular pool that is in dynamic equilibrium with an extra vascular pool. The two-pool model describes a system where an irreversible and reversible clearance routes exist (102). The tracer clearance curve from the PI-injected rabbit was atypical for a two-pool model as the values after the inflection point (3 to 24 hour(s)) resulted in a slope that was greater than zero. This indicated that [^{3}H]-tracer returned to the intravascular pool at a greater rate than it was cleared. Clearly, cholesterol is a difficult metabolite to model in an *in vivo* system due to the conversion to the CE molecule. An approximation on the initial clearance of the tracer was measured by calculating the initial slope of the curve before the inflection point. The initial clearance rate is a reflection of the irreversible tracer clearance. A less

sophisticated approach can be used to characterize differences in the clearance rates by examining the plasma tracer levels at each time-point. The plasma samples from the PI-injected rabbits at the 30-minute, 1, and 3 hour time points showed significantly less tracer than in the plasma of the PC-injected rabbits. These data suggest that the cholesterol tracer was cleared more rapidly in the PI-injected rabbit than the PC-injected rabbit. The use of the initial time point as representative of 100% was used to adjust for slight variations in rabbit blood volumes, and injection of the tracer.

The injected lipid/tracer vesicles are unstable and should rapidly associate with plasma lipoproteins *in vivo*. However, there was a possibility the clearance of radioactivity from plasma may have reflected the clearance rates of the free PI/[^{3}H]-FC and PC/[^{3}H]-FC vesicle complexes. This did not appear to be the case as the majority (75-90%) of the initial radioactivity in the plasma was found associated with the HDL- and LDL-pools in both PI- and PC-injected rabbits. This measurement was only performed once and therefore must be repeated to verify this conclusion. The vesicle-complexes that may exist would be co-isolated with the VLDL-fraction. Therefore, the increased [^{3}H]-FC clearance in the PI-injected rabbits was more likely a result of the rapid clearance from the lipoproteins and not from an enhanced vesicle clearance. When individual lipoprotein clearance was examined in preliminary experiments, the enhanced clearance of [^{3}H]-FC by PI was attributed to the clearance from both HDL and LDL (Figure 7). In general, the data suggest that the clearance rate of [^{3}H]-FC was greater in HDL than in LDL in both PI- and PC-injected rabbits. This experiment should be repeated to verify these results.

The [^{3}H]-FC clearance pathway from the lipoproteins may occur through a conversion to CE or by enhanced tissue uptake. Our data suggested that this enhanced [^{3}H]-FC clearance in the rabbit was not through a conversion to [^{3}H]-CE. We showed that the plasma from the PI-injected rabbits was unable to esterify exogenously added [^{3}H]-FC to [^{3}H]-CE (Figure 8) in the measurements observed from one rabbit. The inhibition of esterification by PI was maximal after 10 minutes and gradually returned to near normal levels after 6 hours post-injection. The cholesterol esterification rate of the PC-injected rabbit was not obviously affected over the 6-hour time period. The LCAT inhibition pattern over time coincided with the lipoprotein surface potential shown in Figure 5. This experiment was performed in only one PI-injected and one PC-injected rabbit and therefore must be repeated to make conclusions on these results. However, subsequent experiments in Dr. Spark's laboratory have confirmed these finding. To verify that the PI is directly inhibiting LCAT, *in vitro* studies were performed. Human and rabbit plasma enriched with PI caused a complete inhibition of LCAT when compared to control plasma. When equivalent amounts of PC was added to plasma no effect on the esterification activity was seen in rabbit plasma, although a 50% increase was observed with human plasma (Figure 9). These results showed that PI caused an inhibition of the enzymatic activity of LCAT, which was in agreement with previous studies in this laboratory (76). This suggested that anionic charge may affect LCAT and that high levels of PI in lipoprotein particles will inhibit cholesterol esterification. The interaction and activation of LCAT by apoA-I on HDL are thought to be electrostatic in nature, and therefore changes in lipoprotein surface charge may disrupt this interaction. Alternatively, as suggested by Sparks *et al,* it may be possible that high levels of PI in

lipoproteins inhibits LCAT activity by dramatically enhancing the binding affinity of LCAT, thus inhibiting its ability to release and bind elsewhere on the particle (76). The inhibitory effect of PI on LCAT may explain the complication observed in the analysis of the clearance curve of the cholesterol from the PI-injected rabbits (Figure 6).

As mentioned previously, there are two possible routes for [^{3}H]-FC to be cleared from the plasma compartment. FC can be directly transferred to the tissue or FC can be converted to CE and then transferred to the tissue. The inhibition of LCAT by PI indicated that PI must stimulate a FC tissue clearance route in the rabbits. The liver is a major route of cholesterol clearance *in vivo* (212-215). Preliminary experiments evaluated [^{3}H]-FC transport into the liver and bile of rabbits injected with PI- and PC-vesicles. These results suggest that the liver may be a route for the PI-enhanced cholesterol clearance from the plasma. There was ~50% more FC tracer observed in the liver of the rabbit injected with PI as compared with the PC-injected rabbit, and an even more pronounced increase in [^{3}H]-FC in the bile of the PI-injected rabbit. This suggests that PI may also increase the transport of FC through the hepatocyte into the bile. In order to conclude that the liver plays a major role in the PI–stimulated clearance of cholesterol from the plasma, additional rabbit experiments must examine uptake by other tissues in addition to the liver. It is possible that the PI-vesicle injection will stimulate an enhanced clearance of FC into the extra-hepatic tissue in addition to the hepatic tissue.

To evaluate the specific role of HDL in FC-clearance by the liver and secretion into the bile, PI- or PC-enriched HDL labelled with [^{3}H]-FC was injected into the rabbits. Preliminary results show that the [^{3}H]-uptake by the liver was similar in the PI-enriched HDL injected rabbit, although ~3.6-fold more [^{3}H] was found in the bile of these rabbits.

This indicates that the PI-enriched HDL may provide a more efficient transport into the bile as compared to the PC-enriched HDL. Therefore, it appears that while HDL may promote FC transport to bile, the other lipoproteins, LDL and VLDL, may promote a hepatic storage of cholesterol. This experiment was only performed once and must be repeated to make proper conclusions.

We also demonstrated that the mass of cholesterol excreted into the faeces may have been affected by the injection of PI. These rabbits were allowed free access to food and water through out the 6-day period. This was to ensure that the daily faecal output remained relatively constant. The data show that over a 6-day period there was no obvious consistent change in the levels of bile acids or cholesterol in the faeces of the rabbits injected with PC-vesicles. However, there appeared to be a 1.7- to 2.4-fold increase (from day 1 to 2) in the cholesterol mass in the faeces of the two PI-injected rabbits (Figure 10). In contrast, the excretion of total bile acids into the faeces of the PI-injected rabbits showed no observable trend. In order for proper statistical analysis to be performed and conclusions to be drawn, this experiment should be repeated to evaluate if the day-to-day variations observed in daily faecal cholesterol and bile acid output was significantly greater than expected by chance. However, subsequent results from the laboratory of Dr. Sparks have been in agreement with results from this thesis. The estimated period that it would take for the rabbit's digestive system to transport a molecule of FC from the plasma into the intestines and into the faeces is approximately 24 hours (216). FC is thought to be the major excretory form of sterol from the reverse cholesterol transport pathway (153; 217). Rigotti *et al* have shown using SR-BI over-expressing mice, that only the biliary cholesterol levels, are increased as a result of the

stimulated reverse cholesterol transport pathway in mice (153). Therefore, PI could stimulate a reverse cholesterol transport pathway, where plasma FC is cleared through a liver, bile, and faecal excretion route. Further experiments are needed to confirm these findings.

It is possible that PI stimulates the clearance of plasma FC by the direct inhibition of LCAT activity. This inhibition, which causes the FC to remain in its free form, may allow it to be taken up at a greater rate by the tissues. Preliminary studies suggest that the clearance of FC from the plasma is a result of an HDL clearance pathway. HDL-FC may be more accessible for uptake by the liver than HDL-CE due to its less hydrophobic structure. Studies have also shown that HDL-FC is preferentially utilised for biliary cholesterol secretion in man (167; 220). This is in agreement with the view that HDL-FC is the transport pool while the cholesterol found in LDL (mainly CE) constitutes the major plasma storage pool (123). Despite their decreased HDL levels, LCAT deficient patients do not have a major increased risk of developing atherosclerosis (120). These patients may be able to efflux cellular cholesterol effectively to the apoB-containing lipoproteins, in the absence of spherical HDL. LCAT deficient mice have also significantly reduced diet-induced aortic atherosclerosis (118; 119). The data suggest that a high LCAT activity may not be required for an effective anti-atherogenic reverse cholesterol transport function.

It was notable that the analysis of lipoprotein lipid and protein mass levels revealed that the PI-injection had no conclusive effect on the lipid compositions in HDL and LDL or VLDL. We expected that the lipoprotein FC levels would decrease dramatically if the clearance of FC were increased. However, because the *in vivo* system

is in dynamic homeostasis, HDL may be able to compensate for the transient enhanced clearance of FC and absorb FC from other tissues. The conclusions based on the data acquired from Table IV must be carefully made due to the large variations between rabbits. The HDL levels appeared to be relatively similar in the four PI-injected rabbits, and showed no significant alterations in lipid levels. The use of radioactive tracer must be interpreted with caution as these values represent the flux and not the net movement of cholesterol mass. It is therefore possible that repetitive daily injections of PI-vesicles in rabbits will cause a significant change in HDL and LDL lipid compositions. Further investigations using the fast protein liquid chromatography (FPLC) method to separate lipoproteins would provide several advantages over the current method used. The FPLC method allows for the separation of plasma lipid levels with very small volume of sample and may allow for the detection of changes in lipid levels in the numerous different lipoprotein fractions. For example, the ultracentrifugation method used to separate the lipoproteins in this thesis does not differentiate between the different HDL classes, and therefore lipid alterations in a particular subtype of HDL may have been over-looked. Subsequent experiments in this laboratory using the FLPC technique suggested that repetitive injections of PI-vesicles in cholesterol fed rabbits over nine days, lowered LDL, and VLDL cholesterol levels when compared to control rabbits, but did not lower HDL cholesterol levels.

In order to evaluate the role of the liver cells in FC-uptake and clearance, a cell culture system was developed to examine the effects of PI in the uptake of cholesterol from plasma and lipoproteins. Since it is thought that the liver is responsible for the majority of the clearance of cholesterol from the blood stream, the HepG2 cell line was

utilised to model hepatocyte function. Studies have shown that these cells metabolise lipoproteins in a similar manner to primary hepatocytes (222-226). Cholesterol uptake by HepG2 cells was examined from plasma that was enriched with 0.2 mg/ml PI or PC vesicles. These phospholipid concentrations were approximated from the levels of PI in the rabbit plasma immediately following vesicle-injection. The phospholipid enrichment of plasma was performed at 4°C to eliminate the possibility of the FC being converted to CE by endogenous LCAT activity. The results show that the uptake of FC from the PI-enriched plasma was enhanced two-fold when compared with the PC control after 1 hour at 37°C (Figure 11B). This was in agreement to the *in vivo* observations and suggested that PI-enhanced FC-clearance occurred by a stimulation of hepatocyte uptake. This PI-stimulated uptake was found to be specifically associated with HDL-FC and not with LDL-FC. FC-uptake studies from LDL indicated that PI-enrichment had no significant effect on cholesterol uptake when compared to PC-enriched LDL. Since we saw similar enhanced uptake patterns with HDL and plasma, we can conclude that HDL in the plasma facilitates the PI-enhanced FC-uptake in HepG2 cells (Figure 13). These findings are not consistent with the preliminary data from the rabbit studies, where the injection of PI caused an enhanced clearance of [^{3}H]-FC from both HDL and LDL when compared to the PC-injected controls. It is possible that the observed PI-enhanced FC-clearance from LDL may be specific to the rabbit model, or alternatively the HepG2 cells may be deficient in the required receptor or transporter to mediate this LDL-FC uptake.

PI specifically enhanced the cellular uptake of cholesterol from HDL in HepG2 cells. Increased FC uptake due to PI-enrichment also appeared to be associated with an enhanced cell association of HDL protein in an experiment performed once. HDL

protein cell association was increased by only ~20% when enriched with PI (Figure 14). The comparison of the protein- and FC-uptake values indicated that the cellular uptake was selective for cholesterol. The initial FC: HDL ratio (mass: mass) was 1:10, while the HepG2 cells absorbed 4-fold more HDL-FC than HDL-protein. We originally thought that the PI-stimulated FC-uptake pathway might involve the major HDL receptor in hepatocytes, SR-BI. Earlier work showed that SR-BI was able to effectively bind anionic lipids and that these vesicles displaced LDL from the receptor (146). It would follow that an HDL molecule enriched with anionic lipids (i.e., PI), would bind to this receptor, and allow a greater flux of cholesterol to the cell. This would also explain the results from the preliminary rabbit experiments as SR-BI has been implicated in the delivery of HDL-FC into the bile for excretion in the faeces. Previous studies have shown that HepG2 cells do express SR-BI (unpublished data by Harder and McPherson, 2001). However, FC-uptake studies utilising the ldlA7 and ldlA7-SR-BI cells suggests that PI does not stimulate an SR-BI uptake pathway (Figure 15). If SR-BI was directly involved in binding PI-HDL, a greater PI-enhanced FC-uptake should be observed in the over-expressing cells. Cellular CE-uptake studies from plasma show that the PI-stimulated pathway is specific for FC. An experiment using [^{3}H]-CE labelled plasma showed that PI did not enhance the uptake of this lipid in HepG2 cells (Figure 12). This again suggests that SR-BI is not involved in this process as it is well documented that SR-BI promotes the selective uptake of CE (149).

The SR-BI cell studies suggest that there may be an alternative pathway involved in the FC-uptake in HepG2 cells, which is specific for HDL-FC. Silver *et al* evaluated the role of cholesterol uptake by SR-BI in hepatocytes, and could not confirm that the

entire sterol uptake was via SR-BI and therefore suggested the existence of an additional FC-uptake pathway in the liver (152). Experiments were performed to identify this cellular FC-uptake mechanism. It has also been reported that another membrane transporter found in the ABC family, MDR1, promotes the uptake of FC from micelles in transfected intestinal cells (28). This transporter is similar in structure to the ABCA-I transporter, which has been shown to be involved in cholesterol transport. The physiological function of MDR1 is not known, but it is highly expressed in steroidal tissues and has been shown to possess a cholesterol-binding site (231). A well-characterized function of MDR1 is the non-specific removal of intra-cellular hydrophobic drugs, an action resulting in cellular drug resistance. MDR1 has been shown to be expressed in the HepG2 cell line at the mRNA and protein level (83). We have therefore attempted to implicate a role for MDR1 in the PI-stimulated HDL-FC uptake by utilising different inhibitors and activators that are known to modulate the activity of the transporter. However, the modulators that have been used in this study are not specific to the MDR1 transporter and therefore provide little evidence of its involvement. In addition, the inhibitors and activators used in this study have been previously shown to activate the MDR1 hydrophobic drug efflux activity, which may have little to do with the proposed role in cholesterol uptake. Therefore, because no specific studies have been performed to alter MDR1 expression levels, modulator studies only provide insight into the potential cell-signalling pathways that may be involved in FC-uptake from HDL.

We evaluated the ability of several chemical cell-signalling modulators to alter cholesterol metabolism in HepG2 cells. Previous work has shown that PKC inhibition will inhibit MDR1 function, while PKC activation can stimulate MDR1. Our results

show that chelerythrine chloride, a PKC inhibitor, inhibits the PI-stimulated FC-uptake by ~50%. Chelerythrine also inhibits the PC-enriched HDL-FC uptake to by ~25% (Figure 16). This suggests that the PKC pathway may be involved in the PI-stimulated FC-uptake pathway and that it may be involved in the basal uptake of HDL-FC. When experiments were performed in the presence of the PKC activator, PMA, no FC-uptake stimulation was observed, suggesting that PI-HDL does not simply activate PKC, but possibly performs other functions, which may be PKC-dependent.

Propranolol has previously been used to inhibit MDR1 function in cell culture experiments (232; 233). Propranolol caused a ~60% percent inhibition of FC-uptake from the PI-enriched HDL and PC-enriched HDL (Figure 16). The fact that propranolol inhibited FC-uptake from both PI- and PC-HDL, suggests that these pathways have common features. Both chelerythrine chloride and propranolol are known to inhibit a common pathway involved in the activation of PKC. The PKC intracellular signalling cascade begins with an activation of phospholipase C (PLC), which hydrolyses intracellular PC. This reaction results in the release of choline-phosphate and diacylglycerol, a lipid that directly activates the PKC pathway by allowing PKC to bind to the inner leaflet of the plasma membrane (234; 235). Chelerythrine chloride directly interacts and inhibits the catalytic domain of PKC. Another source of diacylglycerol is through the action of phospholipase D (PLD), which liberates choline and phosphatidic acid from phospholipids. Phosphatidic acid is hydrolysed to form diacylglycerol, through the action of phosphatidic acid-phosphohydrolase (PAPH). Propranolol can selectively inhibit PAPH activity, decrease diacylglycerol formation, and inhibit PKC activation (236). Therefore, our results suggest that cholesterol uptake may indirectly involve the

PLD activation pathway. It remains unclear whether the MDR1 transporter is involved in a FC-uptake pathway and specific studies must be performed to address this issue.

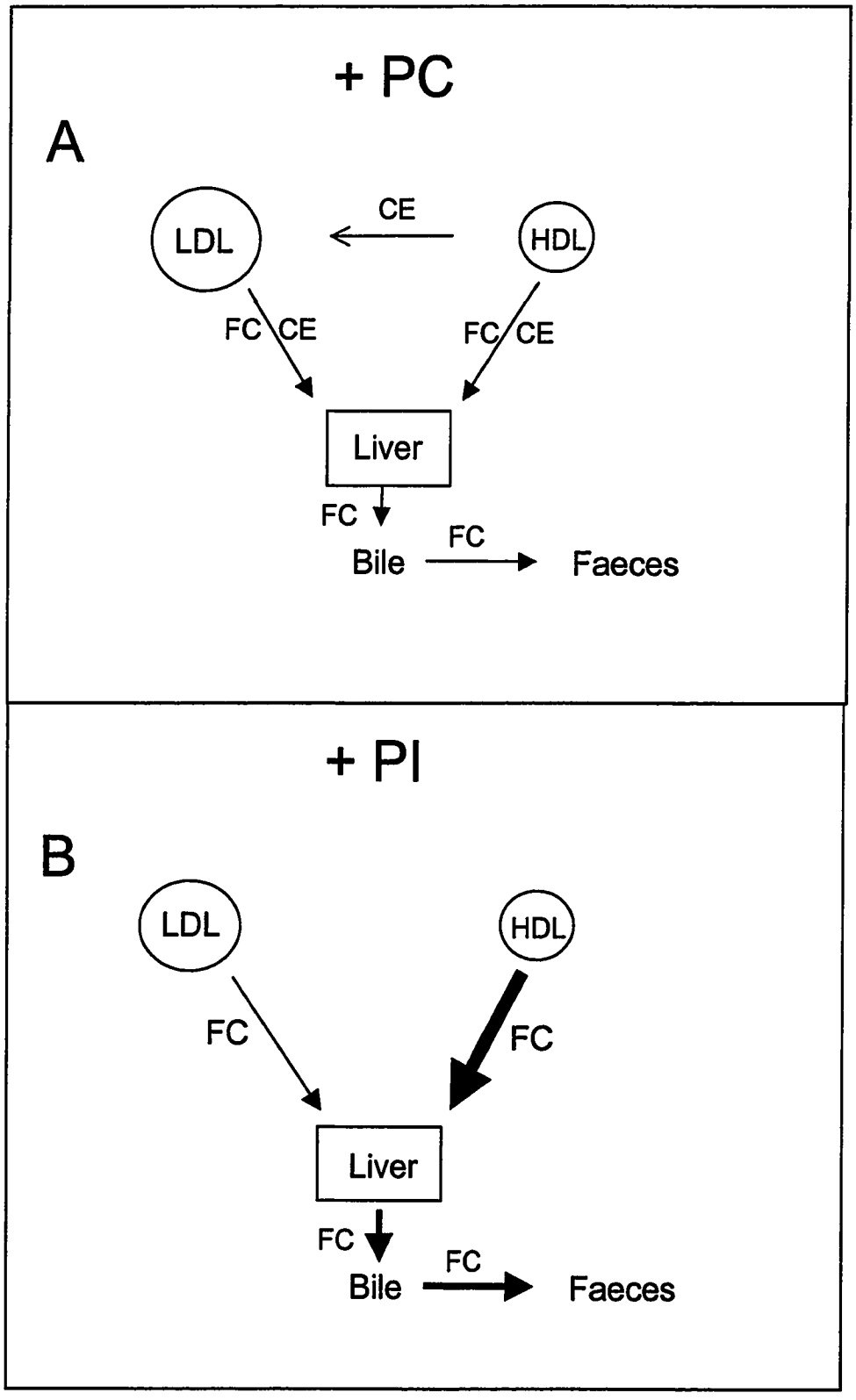
It is important to access at this point if the effects observed are PI specific or anionic lipid specific. PI may enter into the cell independently of HDL and activate this PKC signalling pathway or a FC-uptake mechanism. Prior work has shown that the addition of PI to cells can cause an activation of PKC activity (237). The activation of the PKC has also been linked to the cellular selective uptake of CE from LDL (238). However, our experiments show that when cells are pre-treated with PI-vesicles, FC-uptake from native HDL was unaffected (Figure 17), suggesting that PI-vesicles must be first pre-incubated with the HDL prior to cellular incubation in order to stimulate FC uptake. This may suggest that it is the alteration in HDL surface charge that promotes the increased FC-uptake. It is also possible that the PI requires HDL or apoA-I as a carrier or ligand to enter into the cell or that the presence of HDL components or apoA-I must bind to a receptor to facilitate the PI-response. HDL and apoA-I have previously been shown to act as intracellular-signalling molecules that coordinate cholesterol homeostasis (239-242). It is therefore possible that PI acts in concert with an HDL particle to stimulate FC-uptake by liver cells and that this uptake pathway involves a PKC signalling pathway. Additional studies must be performed with other anionic lipids (i.e. PS) to address if the observed effects are due to the anionic properties of PI or to the specific PI cell-signalling actions.

Summary

Our results show that the injection of a small amount of an anionic lipid into rabbits directly affects the metabolism of cholesterol. The injection of PI-vesicles may

have caused a cascade of events, which eventually promoted cholesterol clearance (Figure 18). The initial step was an increase of the negative lipoprotein surface potential of the three main groups of lipoproteins. This anionic environment appeared to cause an accelerated clearance of the cholesterol tracer from the intravascular system into the tissue when compared to PC-injected control rabbits. Preliminary data suggested that PI might enhance cholesterol uptake by the liver, secretion into the bile and output into the faeces. Additional studies are required to confirm the role of the liver and to characterise other possible tissue uptake pathways involved in the PI-enhanced clearance of cholesterol from the rabbit. These data showed that PI could inhibit LCAT activity and therefore decreased the production of CE in the blood. Cell culture studies suggested that PI stimulated the selective uptake of HDL-FC, (but not LDL-FC) in a human hepatoma cell line. The FC-uptake pathway from HDL appeared to be sensitive to the PKC and PAPH inhibitors. The influence that this anionic lipid has on cholesterol metabolism may provide an important therapeutic intervention that could help in the prevention of atherosclerosis and may provide insight into what factors regulate cholesterol levels in the plasma.

Figure 18. **A speculative model for the effects of a PI-vesicle injection on plasma cholesterol metabolism in rabbits.** Phosphatidylcholine (Panel A) and phosphatidylinositol (Panel B) vesicles were injected into rabbits. Diagram shows the effects of phospholipids on cholesterol clearance. The PI-injection may enhance a lipoprotein mediated FC excretion pathway by the liver.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OTHER ABSTRACTS

- **Stamler CJ** and Sparks DL. Lipoprotein Surface Charge Targets Cholesterol to the Liver. 5th Annual University of Ottawa Heart Institute Research Day. April 2001 (Oral presentation)

- **Stamler CJ** and Sparks DL. Lipoprotein Surface Charge Regulates Cellular Cholesterol Uptake. 25th Annual Canadian Lipoprotein Conference. White Rock, British Columbia, October 2000 (Poster Presentation)

- **Stamler CJ** and Sparks DL. Phosphatidylinositol promotes cholesterol transport *in vivo.* 4th Annual University of Ottawa Heart Institute Research Day. April 2000 (Oral Presentation)

- **Stamler CJ** and Sparks DL. Phosphatidylinositol Promotes Cholesterol Transport *in vivo.* 24th Annual Canadian Lipoprotein Conference. Mt Tremblant, Quebec, October 1999 (Poster Presentation)

- **Stamler CJ** and Sparks DL. Phosphatidylinositol Promotes Cholesterol Transport *in vivo.* University of Ottawa Annual Department of Pathology Conference, March 2000 (Poster Presentation)

- **Stamler CJ** and Sparks DL. . Phosphatidylinositol Promotes Cholesterol Transport *in vivo.* University of Ottawa, Annual Department of Biochemistry, Microbiology and Immunology Poster Day. March 2000

REFERENCES

(1) Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993; 362(6423):801-809.

(2) Lusis AJ. Atherosclerosis. Nature 2000; 407(6801):233-241.

(3) Navab M, Berliner JA, Watson AD, Hama SY, Territo MC, Lusis AJ, Shih DM, Van Lenten BJ, Frank JS, Demer LL, Edwards PA, Fogelman AM. The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. Arterioscler Thromb Vasc Biol 1996; 16(7):831-842.

(4) Glass CK, Witztum JL. Atherosclerosis. The road ahead. Cell 2001; 104(4):503-516.

(5) Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993; 362(6423):801-809.

(6) Navab M, Berliner JA, Watson AD, Hama SY, Territo MC, Lusis AJ, Shih DM, Van Lenten BJ, Frank JS, Demer LL, Edwards PA, Fogelman AM. The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. Arterioscler Thromb Vasc Biol 1996; 16(7):831-842.

(7) Brown MS, Goldstein JL. Regulation of the activity of the low density lipoprotein receptor in human fibroblasts. Cell 1975; 6(3):307-316.

(8) Pike LJ, Casey L. Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains. J Biol Chem 1996; 271(43):26453-26456.

(9) Hamilton JA, Bhamidipati SP, Kodali DR, Small DM. The interfacial conformation and transbilayer movement of diacylglycerols in phospholipid bilayers. J Biol Chem 1991; 266(2):1177-1186.

(10) Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci U S A 1979; 76(1):333-337.

(11) Dhaliwal BS, Steinbrecher UP. Scavenger receptors and oxidized low density lipoproteins. Clin Chim Acta 1999; 286(1-2):191-205.

(12) Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. J Biol Chem 1993; 268(16):11811-11816.

(13) Sambrano GR, Steinberg D. Recognition of oxidatively damaged and apoptotic cells by an oxidized low density lipoprotein receptor on mouse peritoneal macrophages: role of membrane phosphatidylserine. Proc Natl Acad Sci U S A 1995; 92(5):1396-1400.

(14) Brown MS, Goldstein JL. Scavenger cell receptor shared. Nature 1985; 316(6030):680-681.

(15) Brown MS, Goldstein JL. Atherosclerosis. Scavenging for receptors. Nature 1990; 343(6258):508-509.

(16) Carlson LA. Plasma lipids and atherosclerosis. J Clin Pathol Suppl (Assoc Clin Pathol) 1973; 5:43-47.

(17) Carlson LA, Bottiger LE. Ischaemic heart-disease in relation to fasting values of plasma triglycerides and cholesterol. Stockholm prospective study. Lancet 1972; 1(7756):865-868.

(18) Smith EB. The relationship between plasma and tissue lipids in human atherosclerosis. Adv Lipid Res 1974; 12(0):1-49.

(19) Gordon DJ. Epidemiology of lipoproteins. In: Betteridge DJ, Illingworth DR, Shepherd J, editors. Lipoproteins in Health and Disease. New York: Oxford University Press, 1999: 587-595.

(20) Chait A, Heinecke JW. Lipoproteins, Modified Lipoproteins and Atheroscleotiv Vascular Disease. In: Betteridge DJ, Illingworth DR, Shepherd J, editors. Lipoproteins in Health and Disease. New York: Oxford University Press Inc, 1999: 597-610.

(21) Cullis P, Fenske DB, Hope M.J. Physical properties and functional roles of lipids in membranes. In: Vance DE, Vance JE, editors. Biochemistry of Lipids, Lipoproteins adn Membranes. New York: Elsevier, 1996: 1-11.

(22) Acalovschi M. Cholesterol gallstones: from epidemiology to prevention. Postgrad Med J 2001; 77(906):221-229.

(23) Grundy SM. Mechanism of cholesterol gallstones formation. Semin Liver Dis 1983; 3(2):97-111.

(24) Grundy SM. Absorption and metabolism of dietary cholesterol. Annu Rev Nutr 1983; 3:71-96.

(25) Mardones P, Quinones V, Amigo L, Moreno M, Miquel JF, Schwarz M, Miettinen HE, Trigatti B, Krieger M, VanPatten S, Cohen DE, Rigotti A. Hepatic cholesterol and bile acid metabolism and intestinal cholesterol absorption in scavenger receptor class B type I-deficient mice. J Lipid Res 2001; 42(2):170-180.

(26) Lobaccaro JM, Repa JJ, Lu TT, Caira F, Henry-Berger J, Volle DH, Mangelsdorf DJ. [Regulation of lipid metabolism by the orphan nuclear receptors] Regulation du metabolisme des lipides par les recepteurs nucleaires orphelins. Ann Endocrinol (Paris) 2001; 62(3):239-247.

(27) Repa JJ, Turley SD, Lobaccaro JA, Medina J, Li L, Lustig K, Shan B, Heyman RA, Dietschy JM, Mangelsdorf DJ. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. Science 2000; 289(5484):1524-1529.

(28) Tessner TG, Stenson WF. Overexpression of MDR1 in an intestinal cell line results in increased cholesterol uptake from micelles. Biochem Biophys Res Commun 2000; 267(2):565-571.

(29) Edwards P, Davis R. Isoprenoids, sterols and bile acids. In: Vance DE, Vance JE, editors. Biochemistry of Lipids, Lipoproteins and Membranes. New York: Elsevier, 1996: 341-362.

(30) Ikonen E, Parton RG. Caveolins and cellular cholesterol balance. Traffic 2000; 1(3):212-217.

(31) Simons K, Ikonen E. How cells handle cholesterol. Science 2000; 290(5497):1721-1726.

(32) Goldstein JL, Brown MS. The LDL receptor and the regulation of cellular cholesterol metabolism. J Cell Sci Suppl 1985; 3:131-137.

(33) Rudel LL, Lee RG, Cockman TL. Acyl coenzyme A: cholesterol acyltransferase types 1 and 2: structure and function in atherosclerosis. Curr Opin Lipidol 2001; 12(2):121-127.

(34) Chang TY, Chang CC, Cheng D. Acyl-coenzyme A: Cholesterol acyltransferase. Annu Rev Biochem 1997; 66:613-638.

(35) Colbran RJ, Garton AJ, Cordle SR, Yeaman SJ. Regulation of cholesterol ester hydrolase by cyclic AMP-dependent protein kinase. FEBS Lett 1986; 201(2):257-261.

(36) Schlegel A, Lisanti MP. The caveolin triad: caveolae biogenesis, cholesterol trafficking, and signal transduction. Cytokine Growth Factor Rev 2001; 12(1):41-51.

(37) Ostermeyer AG, Paci JM, Zeng Y, Lublin DM, Munro S, Brown DA. Accumulation of caveolin in the endoplasmic reticulum redirects the protein to lipid storage droplets. J Cell Biol 2001; 152(5):1071-1078.

(38) Fielding CJ, Fielding PE. Intracellular cholesterol transport. J Lipid Res 1997; 38(8):1503-1521.

(39) Shen BW, Scanu AM, Kezdy FJ. Structure of human serum lipoproteins inferred from compositional analysis. Proc Natl Acad Sci U S A 1977; 74(3):837-841.

(40) Edelstein C, Kezdy FJ, Scanu AM, Shen BW. Apolipoproteins and the structural organization of plasma lipoproteins: human plasma high density lipoprotein-3. J Lipid Res 1979; 20(2):143-153.

(41) Havel RJ, Goldstein JL, Brown MS. Lipoproteins and lipid transport. In: Bondy PK, Risenberg LE, editors. Metabolic Control and Disease. Philadelphia, PA: W.B. Saunders, 1980: 398.

(42) Alaupovic P, Lee DM, McConathy WJ. Studies on the composition and structure of plasma lipoproteins. Distribution of lipoprotein families in major density classes of normal human plasma lipoproteins. Biochim Biophys Acta 1972; 260(4):689-707.

(43) Coffill CR, Ramsamy TA, Hutt DM, Schultz JR, Sparks DL. Diacylglycerol is the preferred substrate in high density lipoproteins for human hepatic lipase. J Lipid Res 1997; 38(11):2224-2231.

(44) Sparks DL, Pritchard PH. Transfer of cholesteryl ester into high density lipoprotein by cholesteryl ester transfer protein: effect of HDL lipid and apoprotein content. Journal of Lipid Research 1989; 30:1491-1498.

(45) Sparks DL, Anantharamaiah GM, Segrest JP, Phillips MC. Effect of the cholesterol content of reconstituted LpA-I on lecithin:cholesterol acyltransferase activity. J Biol Chem 1995; 270(10):5151-5157.

(46) Sparks DL, Frank PG, Neville TA. Effect of the surface lipid composition of reconstituted LPA-I on apolipoprotein A-I structure and lecithin: cholesterol acyltransferase activity. Biochim Biophys Acta 1998; 1390(2):160-172.

(47) Masson D, Athias A, Lagrost L. Evidence for electronegativity of plasma high density lipoprotein-3 as one major determinant of human cholesteryl ester transfer protein activity. J Lipid Res 1996; 37(7):1579-1590.

(48) Fredrickson DS, Levy RI, Lees RS. Fat transport in lipoproteins--an integrated approach to mechanisms and disorders. N Engl J Med 1967; 276(1):34-42.

(49) Noble RP. Electrophoretic separation of plasma lipoproteins in agarose gel. J Lipid Res 1968; 9(6):693-700.

(50) Sparks DL, Phillips MC. Quantitative measurement of lipoprotein surface charge by agarose gel electrophoresis. J Lipid Res 1992; 33(1):123-130.

(51) Desrumaux C, Athias A, Masson D, Gambert P, Lallemant C, Lagrost L. Influence of the electrostatic charge of lipoprotein particles on the activity of the human plasma phospholipid transfer protein. Journal of Lipid Research 1998; 39(1):131-142.

(52) Masson D, Athias A, Lagrost L. Evidence for electronegativity of plasma high density lipoprotein-3 as one major determinant of human cholesteryl ester transfer protein activity. Journal of Lipid Research 1996; 37(7):1579-1590.

(53) Sparks DL, Lund-Katz S, Phillips MC. The charge and structural stability of apolipoprotein A-I in discoidal and spherical recombinant high density lipoprotein particles. J Biol Chem 1992; 267(36):25839-25847.

(54) Sparks DL, Davidson WS, Lund-Katz S, Phillips MC. Effects of the neutral lipid content of high density lipoprotein on apolipoprotein A-I structure and particle stability. J Biol Chem 1995; 270(45):26910-26917.

(55) Davidson WS, Sparks DL, Lund-Katz S, Phillips MC. The molecular basis for the difference in charge between pre-beta- and alpha-migrating high density lipoproteins. J Biol Chem 1994; 269(12):8959-8965.

(56) Miller NE, Nanjee MN. Evidence that reverse cholesterol transport is stimulated by lipolysis of triglyceride-rich lipoproteins. FEBS Lett 1991; 285:132-134.

(57) Holub BJ. The nutritional significance, metabolism, and function of myo-inositol and phosphatidylinositol in health and disease. Adv Nutr Res 1982; 4:107-141.

(58) Holub BJ. 1982 Borden Award lecture. Nutritional, biochemical, and clinical aspects of inositol and phosphatidylinositol metabolism. Can J Physiol Pharmacol 1984; 62(1):1-8.

(59) Leach AB, Holub BJ. The effect of dietary lipid on the lipoprotein status of the Mongolian gerbil. Lipids 1984; 19(1):25-33.

(60) Nilsson A, Chen Q, Dahlman E. Metabolism of chylomicron phosphatidylinositol in the rat: fate in vivo and hydrolysis with lipoprotein lipase and hepatic lipase in vitro. J Lipid Res 1994; 35(12):2151-2160.

(61) Ishida T, Koba K, Sugano M, Imaizumi K, Watanabe S, Minoshima R. Cholesterol levels and eicosanoid production in rats fed phosphatidylinositol or soybean lecithin. J Nutr Sci Vitaminol (Tokyo) 1988; 34(2):237-244.

(62) Davis AD, Vance JE. Structure, Assembly and Secretion of Lipoproteins. In: Vance DE, Vance JE, editors. Biochemistry of Lipids, Lipoproteins and Membranes. New York: Elsevier, 1996: 473-493.

(63) Wang CS, Hartsuck J, McConathy WJ. Structure and functional properties of lipoprotein lipase. Biochim Biophys Acta 1992; 1123(1):1-17.

(64) Cohen JC. Chylomicron triglyceride clearance: comparison of three assessment methods. Am J Clin Nutr 1989; 49(2):306-313.

(65) Nicoll A, Miller NE, Lewis B. High-density lipoprotein metabolism. Adv Lipid Res 1980; 17:53-106.

(66) Cladaras C, Hadzopoulou-Cladaras M, Nolte RT, Atkinson D, Zannis VI. The complete sequence and structural analysis of human apolipoprotein B-100: relationship between apoB-100 and apoB-48 forms. EMBO J 1986; 5(13):3495-3507.

(67) Fielding PE, Fielding CJ. Dynamics of Lipoprotein Transport in the Human Circulatory System. In: Vance DE, Vance JE, editors. Biochemistry of Lipids, Lipoproteins and Membranes. New York: Elsevier, 1996: 495-540.

(68) Brown MS, Kovanen PT, Goldstein JL. Regulation of plasma cholesterol by lipoprotein receptors. Science 1981; 212(4495):628-635.

(69) Brown MS, Ho YK, Goldstein JL. The low-density lipoprotein pathway in human fibroblasts: relation between cell surface receptor binding and endocytosis of low-density lipoprotein. Ann N Y Acad Sci 1976; 275:244-257.

(70) Goldstein JL, Anderson RG, Brown MS. Receptor-mediated endocytosis and the cellular uptake of low density lipoprotein. Ciba Found Symp 1982;(92):77-95.

(71) Eisenberg S. High density lipoprotein metabolism. [Review]. Journal of Lipid Research 1984; 25(10):1017-1058.

(72) Glomset JA. The plasma lecithins:cholesterol acyltransferase reaction. [Review]. Journal of Lipid Research 1968; 9(2):155-167.

(73) Green PH, Glickman RM. Intestinal lipoprotein metabolism. J Lipid Res 1981; 22(8):1153-1173.

(74) Small DM. Structure and metabolism of the plasma lipoproteins. In: Kreisberg RA, Segrest JP, editors. Plasma lipoprotein and coronary artery disease. Boston: Blackwell Scientific Publications, 1992: 57-91.

(75) Segrest JP, Jones MK, De Loof H, Brouillette CG, Venkatachalapathi YV, Anantharamaiah GM. The amphipathic helix in the exchangeable apolipoproteins: a review of secondary structure and function. [Review] [118 refs]. Journal of Lipid Research 1992; 33(2):141-166.

(76) Sparks DL, Frank PG, Neville TA. Effect of the surface lipid composition of reconstituted LPA-I on apolipoprotein A-I structure and lecithin: cholesterol acyltransferase activity. Biochim Biophys Acta 1998; 1390(2):160-172.

(77) Sparks DL, Frank PG, Braschi S, Neville TA, Marcel YL. Effect of apolipoprotein A-I lipidation on the formation and function of pre-beta and alpha-migrating LpA-I particles. Biochemistry 1999; 38(6):1727-1735.

(78) Huggins KW, Curtiss LK, Gebre AK, Parks JS. Effect of long chain polyunsaturated fatty acids in the sn-2 position of phosphatidylcholine on the interaction with recombinant high density lipoprotein apolipoprotein A-I. J Lipid Res 1998; 39(12):2423-2431.

(79) Miller GJ, Miller NE. Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease. Lancet 1975; 1(7897):16-19.

(80) Jacobs DR, Jr., Mebane IL, Bangdiwala SI, Criqui MH, Tyroler HA. High density lipoprotein cholesterol as a predictor of cardiovascular disease mortality in men and women: the follow-up study of the Lipid Research Clinics Prevalence Study. Am J Epidemiol 1990; 131(1):32-47.

(81) Francis GA, Mendez AJ, Bierman EL, Heinecke JW. Oxidative tyrosylation of high density lipoprotein by peroxidase enhances cholesterol removal from cultured fibroblasts and macrophage foam cells. Proc Natl Acad Sci USA 1993; 90:6631-6635.

(82) Navab M, Berliner JA, Subbanagounder G, Hama S, Lusis AJ, Castellani LW, Reddy S, Shih D, Shi W, Watson AD, Van Lenten BJ, Vora D, Fogelman AM. HDL and the inflammatory response induced by LDL-derived oxidized phospholipids. Arterioscler Thromb Vasc Biol 2001; 21(4):481-488.

(83) Zhao JY, Ikeguchi M, Eckersberg T, Kuo MT. Modulation of multidrug resistance gene expression by dexamethasone in cultured hepatoma cells. Endocrinology 1993 Aug;133(2):521-8

(84) Pruzanski W, Stefanski E, de Beer FC, de Beer MC, Ravandi A, and Kuksis A. Comparative analysis of lipid composition of normal and acute-phase high density lipoproteins. J. Lipid Res. 2000; 41:1035-1047.

(85) Whitlock ME, Swenson TL, Ramakrishnan R, Leonard MT, Marcel YL, Milne RW, and Tall AR. Monoclonal Antibody inhibition of cholesteryl ester transfer protein activity in the rabbit. J. Clin. Investigations. 1989; 84, 129-137.

(86) Tall AR. An overview of reverse cholesterol transport. Eur Heart J 1998; 19 Suppl A:A31-A35.

(87) Von Eckardstein A, Nofer JR, Assmann G. High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport. Arterioscler Thromb Vasc Biol 2001; 21(1):13-27.

(88) Hill SA, McQueen MJ. Reverse cholesterol transport--a review of the process and its clinical implications. Clin Biochem 1997; 30(7):517-525.

(89) Rothblat GH, Mahlberg FH, Johnson WJ, Phillips MC. Apolipoproteins, membrane cholesterol domains, and the regulation of cholesterol efflux. J Lipid Res 1992; 33(8):1091-1097.

(90) Johnson WJ, Mahlberg FH, Rothblat GH, Phillips MC. Cholesterol transport between cells and high-density lipoproteins. Biochim Biophys Acta 1991; 1085(3):273-298.

(91) Johnson WJ, Mahlberg FH, Chacko GK, Phillips MC, Rothblat GH. The influence of cellular and lipoprotein cholesterol contents on the flux of cholesterol between fibroblasts and high density lipoprotein. J Biol Chem 1988; 263(28):14099-14106.

(92) Karlin JB, Johnson WJ, Benedict CR, Chacko GK, Phillips MC, Rothblat GH. Cholesterol flux between cells and high density lipoprotein. Lack of relationship to specific binding of the lipoprotein to the cell surface. J Biol Chem 1987; 262(26):12557-12564.

(93) Bale PM, Clifton-Bligh P, Benjamin BN, Whyte HM. Pathology of Tangier disease. J Clin Pathol 1971; 24(7):609-616.

(94) Hoffman HN, Fredrickson DS. Tangier disease (familial high density lipoprotein deficiency). Clinical and genetic features in two adults. Am J Med 1965; 39(4):582-593.

(95) Assman G, Eckardstein AV. Tangier Disease. In: Betteridge DL, Illingworth DR, Shepard J, editors. Lipoproteins in Health and Disease. New York: Oxford University Press Inc., 1999: 767-781.

(96) Serfaty-Lacrosniere C, Civeira F, Lanzberg A, Isaia P, Berg J, Janus ED, Smith MP, Jr., Pritchard PH, Frohlich J, Lees RS, . Homozygous Tangier disease and cardiovascular disease. Atherosclerosis 1994; 107(1):85-98.

(97) Schmitz G, Kaminski WE, Orso E. ABC transporters in cellular lipid trafficking. Curr Opin Lipidol 2000; 11(5):493-501.

(98) Remaley AT, Rust S, Rosier M, Knapper C, Naudin L, Broccardo C, Peterson KM, Koch C, Arnould I, Prades C, Duverger N, Funke H, Assman G, Dinger M, Dean M, Chimini G, Santamarina-Fojo S, Fredrickson DS, Denefle P, Brewer HB, Jr. Human ATP-binding cassette transporter 1 (ABC1): genomic organization and identification of the genetic defect in the original Tangier disease kindred. Proc Natl Acad Sci U S A 1999; 96(22):12685-12690.

(99) Costet P, Luo Y, Wang N, Tall AR. Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. J Biol Chem 2000; 275(36):28240-28245.

(100) Li Q, Tsujita M, Yokoyama S. Selective down-regulation by protein kinase C inhibitors of apolipoprotein-mediated cellular cholesterol efflux in macrophages. Biochemistry 1997; 36(40):12045-12052.

(101) Fruchart JC, Staels B, Duriez P. The role of fibric acids in atherosclerosis. Curr Atheroscler Rep 2001 Jan;3(1):83-92

(102) Le NA, Ramakrishnan R, Dell RB, Ginsberg HN, Brown WV. Kinetic analysis using specific radioactivity data. Methods Enzymol. 1986;129:384-95

(103) Wang N, Silver DL, Costet P, Tall AR. Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. J Biol Chem 2000; 275(42):33053-33058.

(104) Fielding PE, Nagao K, Hakamata H, Chimini G, Fielding CJ. A two-step mechanism for free cholesterol and phospholipid efflux from human vascular cells to apolipoprotein A-1. Biochemistry 2000; 39(46):14113-14120.

(105) Vaisman BL, Lambert G, Amar M, Joyce C, Ito T, Shamburek RD, Cain WJ, Fruchart-Najib J, Neufeld ED, Remaley AT, Brewer HB, Jr., Santamarina-Fojo S. ABCA1 overexpression leads to hyperalphalipoproteinemia and increased biliary cholesterol excretion in transgenic mice. J Clin Invest 2001; 108(2):303-309.

(106) Jonas A. Regulation of lecithin cholesterol acyltransferase activity. Prog Lipid Res 1998; 37(4):209-234.

(107) Dobiasova M, Frohlich JJ. Advances in understanding of the role of lecithin cholesterol acyltransferase (LCAT) in cholesterol transport. Clin Chim Acta 1999; 286(1-2):257-271.

(108) Jonas A. Lecithin-cholesterol acyltransferase in the metabolism of high-density lipoproteins. Biochim Biophys Acta 1991; 1084:205-220.

(109) Fielding CJ, Shore VG, Fielding PE. A protein cofactor of lecithin:cholesterol acyltransferase. Biochem Biophys Res Commun 1972; 46(4):1493-1498.

(110) Wang J, DeLozier JA, Gebre AK, Dolphin PJ, Parks JS. Role of glutamic acid residues 154, 155, and 165 of lecithin:cholesterol acyltransferase in cholesterol esterification and phospholipase A2 activities. J Lipid Res 1998; 39(1):51-58.

(111) Jin L, Shieh JJ, Grabbe E, Adimoolam S, Durbin D, Jonas A. Surface plasmon resonance biosensor studies of human wild-type and mutant lecithin cholesterol acyltransferase interactions with lipoproteins. Biochemistry 1999; 38(47):15659-15665.

(112) Glomset JA. Physiological role of lecithin:cholesterol acyltransferase. Am J Clin Nutr 1970; 23:1129-1136.

(113) Glomset JA. The metabolic role of lecithin: cholesterol acyltransferase: perspectives from pathology. Adv Lipid Res 1973; 11:1-65.

(114) Braschi S, Neville TA, Vohl MC, Sparks DL. Apolipoprotein A-I charge and conformation regulate the clearance of reconstituted high density lipoprotein in vivo. J Lipid Res 1999; 40(3):522-532.

(115) Blanco-Vaca F, Qu SJ, Fiol C, Fan HZ, Pao Q, Marzal-Casacuberta A, Albers JJ, Hurtado I, Gracia V, Pintó X, Martí T, Pownall HJ. Molecular basis of fish-eye disease in a patient from Spain - Characterization of a novel mutation in the LCAT gene and lipid analysis of the cornea. Arterioscler Tromb Vasc Biol 1997; 17(7):1382-1391.

(116) Elkhalil L, Majd Z, Bakir R, Perez-Mendez O, Castro G, Poulain P, Lacroix B, Duhal N, Fruchart JC, Luc G. Fish-eye disease: Structural and in vivo metabolic abnormalities of high-density lipoproteins. Metabolism: Clinical and Experimental 1997; 46(5):474-483.

(117) Von Eckardstein A, Huang Y, Wu S, Funke H, Noseda G, Assmann G. Reverse cholesterol transport in plasma of patients with different forms of familial HDL deficiency. Arterioscler Tromb Vasc Biol 1995; 15(5):691-703.

(118) Sakai N, Vaisman BL, Koch CA, Hoyt RF, Jr., Meyn SM, Talley GD, Paiz JA, Brewer HB, Jr., Santamarina-Fojo S. Targeted disruption of the mouse lecithin:cholesterol acyltransferase (LCAT) gene. Generation of a new animal model for human LCAT deficiency. J Biol Chem 1997; 272(11):7506-7510.

(119) Sakai N, Vaisman BL, Koch CA, Paiz JA, Brewer HB, Jr., Santamarina-Fojo S. Analysis of aortic atherosclerosis and glomerulosclerosis. Circulation 96[1], 229. 1997.

(120) Klein HG, Lohse P, Duverger N, Albers JJ, Rader DJ, Zech LA, Santamarina-Fojo S, Brewer HB, Jr. Two different allelic mutations in the lecithin:cholesterol acyltransferase (LCAT) gene resulting in classic LCAT deficiency: LCAT (tyr83-->stop) and LCAT (tyr156-->asn). Journal of Lipid Research 1993; 34(1):49-58.

(121) Amar MJA, Shamburek RD, Foger B, Hoyt Jr RF, Wood DO, Santamarina-Fojo S, Brewer HB, Jr. Expression of murine LCAT in transgenic mice;evaluation of the role of LCAT cholesteryl ester-fatty acid specificity in the development of atherosclerosis. Circulation 98[1], 35. 1998.

(122) Santamarina-Fojo S, Lambert G, Hoeg JM, Brewer HB, Jr. Lecithin-cholesterol acyltransferase: role in lipoprotein metabolism, reverse cholesterol transport and atherosclerosis. Curr Opin Lipidol 2000; 11(3):267-275.

(123) Sparks D, Frohlich JJ, Pritchard PH. Lipid transfer proteins, hypertriglyceridemia, and reduced high-density lipoprotein cholesterol [editorial]. Am Heart J 1991; 122(2):601-607.

(124) Tall A. Plasma lipid transfer proteins. Annu Rev Biochem 1995; 64:235-257.

(125) Nichols AV, Smith L. J.Lipid Res 6, 206-210. 1965.

(126) Lagrost L. Regulation of cholesteryl ester transfer protein (CETP) activity: review of in vitro and in vivo studies. Biochim Biophys Acta 1994; 1215(3):209-236.

(127) Benoist F, Lau P, McDonnell M, Doelle H, Milne R, McPherson R. Cholesteryl ester transfer protein mediates selective uptake of high density lipoprotein cholesteryl esters by human adipose tissue. J Biol Chem 1997; 272(38):23572-23577.

(128) Rinninger F, Pittman RC. Mechanism of the cholesteryl ester transfer protein-mediated uptake of high density lipoprotein cholesteryl esters by Hep G2 cells. J Biol Chem 1989; 264(11):6111-6118.

(129) Granot E, Tabas I, Tall AR. Human plasma cholesteryl ester transfer protein enhances the transfer of cholesteryl ester from high density lipoproteins into cultured HepG2 cells. J Biol Chem 1987; 262(8):3482-3487.

(130) Rittershaus CW, Miller DP, Thomas LJ, Picard MD, Honan CM, Emmett CD, Pettey CL, Adari H, Hammond RA, Beattie DT, Callow AD, Marsh HC, Ryan US. Vaccine-induced antibodies inhibit CETP activity in vivo and reduce aortic lesions in a rabbit model of atherosclerosis. Arterioscler Thromb Vasc Biol 2000; 20(9):2106-2112.

(131) Okamoto H, Yonemori F, Wakitani K, Minowa T, Maeda K, Shinkai H. A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. Nature 2000; 406(6792):203-207.

(132) Tall A, Sharp D, Zhong S, Hayek T, Masucci-Magoulas L, Rubin EM, Breslow JL. Cholesteryl ester transfer protein and atherogenesis. Ann NY Acad Sci 1997; 811:178-184.

(133) Hirano K, Yamashita S, Matsuzawa Y. Pros and cons of inhibiting cholesteryl ester transfer protein. Curr Opin Lipidol 2000; 11(6):589-596.

(134) McPherson R. Function of cholesteryl ester transfer protein in reverse cholesterol transport. Can Med Assoc J 1993; 148:2165-2166.

(135) Bruce C, Chouinard RA, Jr., Tall AR. Plasma lipid transfer proteins, high-density lipoproteins, and reverse cholesterol transport. Annu Rev Nutr 1998; 18:297-330.

(136) Ueda Y, Gong E, Royer L, Cooper PN, Francone OL, Rubin EM. Relationship between expression levels and atherogenesis in scavenger receptor class B, type I transgenics. J Biol Chem 2000; 275(27):20368-20373.

(137) Hammad SM, Barth JL, Knaak C, Argraves WS. Megalin acts in concert with cubilin to mediate endocytosis of high density lipoproteins. J Biol Chem 2000; 275(16):12003-12008.

(138) Moestrup SK, Kozyraki R. Cubilin, a high-density lipoprotein receptor. Curr Opin Lipidol 2000; 11(2):133-140.

(139) Kurata H, Matsumoto A. [The family of HDL receptor]. Nippon Rinsho 1999; 57(12):2704-2710.

(140) Calvo D, Gomez-Coronado D, Suarez Y, Lasuncion MA, Vega MA. Human CD36 is a high affinity receptor for the native lipoproteins HDL, LDL, and VLDL. J Lipid Res 1998; 39(4):777-788.

(141) de Villiers WJ, Cai L, Webb NR, de Beer MC, van Der Westhuyzen DR, de Beer FC. CD36 does not play a direct role in HDL or LDL metabolism. J Lipid Res. 2001 Aug;42(8):1231-8.

(142) Murao K, Terpstra V, Green SR, Kondratenko N, Steinberg D, Quehenberger O. Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes. J Biol Chem 1997; 272(28):17551-17557.

(143) Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science 1996; 271(5248):518-520.

(144) Johnson MS, Svensson PA, Helou K, Billig H, Levan G, Carlsson LM, Carlsson B. Characterization and chromosomal localization of rat scavenger receptor class B type I, a high density lipoprotein receptor with a putative leucine zipper domain and peroxisomal targeting sequence. Endocrinology 1998; 139(1):72-80.

(145) Svensson PA, Johnson MS, Ling C, Carlsson LM, Billig H, Carlsson B. Scavenger receptor class B type I in the rat ovary: Possible role in high density lipoprotein cholesterol uptake and in the recognition of apoptotic granulosa cells. Endocrinology 1999; 140(6):2494-2500.

(146) Rigotti A, Acton SL, Krieger M. The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. J Biol Chem 1995; 270(27):16221-16224.

(147) Rigotti A, Trigatti B, Babitt J, Penman M, Xu S, Krieger M. Scavenger receptor BI--a cell surface receptor for high density lipoprotein. Curr Opin Lipidol 1997; 8(3):181-188.

(148) Xu S, Laccotripe M, Huang X, Rigotti A, Zannis VI, Krieger M. Apolipoproteins of HDL can directly mediate binding to the scavenger receptor SR-BI, an HDL receptor that mediates selective lipid uptake. J Lipid Res 1997; 38(7):1289-1298.

(149) Rigotti A, Trigatti B, Babitt J, Penman M, Xu S, Krieger M. Scavenger receptor BI--a cell surface receptor for high density lipoprotein. Curr Opin Lipidol 1997; 8(3):181-188.

(150) Llera-Moya M, Rothblat GH, Connelly MA, Kellner-Weibel G, Sakr SW, Phillips MC, Williams DL. Scavenger receptor BI (SR-BI) mediates free cholesterol flux independently of HDL tethering to the cell surface. J Lipid Res 1999; 40(3):575-580.

(151) de la Llera-Moya M, Rothblat GH, Connelly MA, Kellner-Weibel G, Sakr SW, Phillips MC, Williams DL. Scavenger receptor BI (SR-BI) mediates free cholesterol flux independently of HDL tethering to the cell surface. J Lipid Res 1999; 40(3):575-580.

(152) Silver DL, Wang N, Xiao X, Tall AR. High density lipoprotein (hdl) particle uptake mediated by scavenger receptor class b type 1 results in selective sorting of hdl cholesterol from protein and polarized cholesterol secretion. J Biol Chem 2001; 276(27):25287-25293.

(153) Kozarsky KF, Donahee MH, Rigotti A, Iqbal SN, Edelman ER, Krieger M. Overexpression of the HDL receptor SR-BI alters plasma HDL and bile cholesterol levels. Nature 1997; 387(6631):414-417.

(154) Kozarsky KF, Donahee MH, Glick JM, Krieger M, Rader DJ. Gene transfer and hepatic overexpression of the HDL receptor SR-BI reduces atherosclerosis in the cholesterol-fed LDL receptor-deficient mouse. Arterioscler Thromb Vasc Biol 2000; 20(3):721-727.

(155) Spady DK. Hepatic clearance of plasma low density lipoproteins. Semin Liver Dis 1992; 12(4):373-385.

(156) Cohen DE. Hepatocellular transport and secretion of biliary lipids. Curr Opin Lipidol 1999; 10(4):295-302.

(157) Hofmann AF. Current concepts of biliary secretion. Dig Dis Sci 1989; 34(12 Suppl):16S-20S.

(158) Bear CE, Shaffer EA. Hepatocellular water and electrolyte secretion. Can J Physiol Pharmacol 1988; 66(10):1253-1260.

(159) Strange RC. Hepatic bile flow. Physiol Rev 1984; 64(4):1055-1102.

(160) Frizzell RA, Heintze K. Transport functions of the gallbladder. Int Rev Physiol 1980; 21:221-247.

(161) Paumgartner G, Sauerbruch T. Secretion, composition and flow of bile. Clin Gastroenterol 1983; 12(1):3-23.

(162) Glickman RM. Fat absorption and malabsorption. Clin Gastroenterol 1983; 12(2):323-334.

(163) Friedman HI, Nylund B. Intestinal fat digestion, absorption, and transport. A review. Am J Clin Nutr 1980; 33(5):1108-1139.

(164) Borgstrom B. Bile salts--their physiological functions in the gastrointestinal tract. Acta Med Scand 1974; 196(1-2):1-10.

(165) Shneider BL. Intestinal bile acid transport: biology, physiology, and pathophysiology. J Pediatr Gastroenterol Nutr 2001; 32(4):407-417.

(166) Chawla A, Saez E, Evans RM. "Don't know much bile-ology". Cell 2000; 103(1):1-4.

(167) Schwartz CC, Halloran LG, Vlahcevic ZR, Gregory DH, Swell L. Preferential utilization of free cholesterol from high-density lipoproteins for biliary cholesterol secretion in man. Science 1978; 200(4337):62-64.

(168) Zegers MM, Hoekstra D. Mechanisms and functional features of polarized membrane traffic in epithelial and hepatic cells. Biochem J 1998; 336(Pt 2):257-269.

(169) Elferink RP, Tytgat GN, Groen AK. Hepatic canalicular membrane 1: The role of mdr2 P-glycoprotein in hepatobiliary lipid transport. FASEB J 1997; 11(1):19-28.

(170) Buschman E, Arceci RJ, Croop JM, Che M, Arias IM, Housman DE, Gros P. mdr2 encodes P-glycoprotein expressed in the bile canalicular membrane as determined by isoform-specific antibodies. J Biol Chem 1992; 267(25):18093-18099.

(171) Erlinger S. Review article: new insights into the mechanisms of hepatic transport and bile secretion. J Gastroenterol Hepatol 1996; 11(6):575-579.

(172) Ortiz D, Arias IM. MDR3 mutations: a glimpse into pandora's box and the future of canalicular pathophysiology. Gastroenterology 2001; 120(6):1549-1552.

(173) Crawford AR, Smith AJ, Hatch VC, Oude Elferink RP, Borst P, Crawford JM. Hepatic secretion of phospholipid vesicles in the mouse critically depends on mdr2 or MDR3 P-glycoprotein expression. Visualization by electron microscopy. J Clin Invest 1997; 100(10):2562-2567.

(174) Sehayek E, Ono JG, Shefer S, Nguyen LB, Wang N, Batta AK, Salen G, Smith JD, Tall AR, Breslow JL. Biliary cholesterol excretion: a novel mechanism that regulates dietary cholesterol absorption. Proc Natl Acad Sci U S A 1998; 95(17):10194-10199.

(175) Fuchs M, Ivandic B, Muller O, Schalla C, Scheibner J, Bartsch P, Stange EF. Biliary cholesterol hypersecretion in gallstone-susceptible mice is associated with hepatic up-regulation of the high-density lipoprotein receptor SRBI. Hepatology 2001; 33(6):1451-1459.

(176) Ji Y, Wang N, Ramakrishnan R, Sehayek E, Huszar D, Breslow JL, Tall AR. Hepatic scavenger receptor BI promotes rapid clearance of high density lipoprotein free cholesterol and its transport into bile. J Biol Chem 1999; 274(47):33398-33402.

(177) Hillebrant CG, Nyberg B, Einarsson K, Eriksson M. The effect of plasma low density lipoprotein apheresis on the hepatic secretion of biliary lipids in humans. Gut 1997; 41(5):700-704.

(178) Lipid Reserach Clinics Program, . The lipid researchclinics coronary primary prevention trial results I. Reduction in incidence of coronary heart disease. Journal of the American Medical Association 251, 351-364. 1984.

(179) Frick MH, Elo O, Haapa K, Heinonen OP, Heinsalmi P, Helo P, Huttunen JK, Kaitaniemi P, Koskinen P, Manninen V, . Helsinki Heart Study: primary-prevention trial with gemfibrozil in middle-aged men with dyslipidemia. Safety of treatment, changes in risk factors, and incidence of coronary heart disease. N Engl J Med 1987; 317(20):1237-1245.

(180) Endo A. The discovery and development of HMG-CoA reductase inhibitors. J Lipid Res 1992; 33(11):1569-1582.

(181) Illingworth DR, Bacon S. Treatment of heterozygous familial hypercholesterolemia with lipid-lowering drugs. Arteriosclerosis 1989; 9(1 Suppl):I121-I134.

(182) Walker JF. HMG CoA reductase inhibitors. Current clinical experience. Drugs 1988; 36 Suppl 3:83-86.

(183) Illingworth DR. An overview of lipid-lowering drugs. Drugs 1988; 36 Suppl 3:63-71.

(184) Beaird SL. HMG-CoA reductase inhibitors: assessing differences in drug interactions and safety profiles. J Am Pharm Assoc (Wash) 2000; 40(5):637-644.

(185) Farmer JA, Torre-Amione G. Comparative tolerability of the HMG-CoA reductase inhibitors. Drug Saf 2000; 23(3):197-213.

(186) Dietz BL, Oberg KC. Judicious evaluation of adverse drug reactions: inaccurate assessment of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor-induced muscle injury. Pharmacotherapy 1999; 19(2):232-235.

(187) von Eckardstein A, Assmann G. Prevention of coronary heart disease by raising high-density lipoprotein cholesterol? Curr Opin Lipidol 2000; 11(6):627-637.

(188) Eriksson M, Carlson LA, Miettinen TA, Angelin B. Stimulation of fecal steroid excretion after infusion of recombinant proapolipoprotein A-I. Potential reverse cholesterol transport in humans. Circulation 1999; 100(6):594-598.

(189) Rodrigueza WV, Mazany KD, Essenburg AD, Pape ME, Rea TJ, Bisgaier CL, Williams KJ. Large versus small unilamellar vesicles mediate reverse cholesterol transport in vivo into two distinct hepatic metabolic pools. Implications for the treatment of atherosclerosis. Arterioscler Thromb Vasc Biol 1997; 17(10):2132-2139.

(190) Alam K, Meidell RS, Spady DK. Effect of up-regulating individual steps in the reverse cholesterol transport pathway on reverse cholesterol transport in normolipidemic mice. J Biol Chem 2001; 276(19):15641-15649.

(191) Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 1978; 87(1):206-210.

(192) Dobiasova M, Stribrna J, Sparks DL, Pritchard PH, Frohlich JJ. Cholesterol esterification rates in very low density lipoprotein- and low density lipoprotein-depleted plasma. Relation to high density lipoprotein subspecies, sex, hyperlipidemia, and coronary artery disease. Arterioscler Thromb 1991; 11(1):64-70.

(193) Glass C, Pittman RC, Civen M, Steinberg D. Uptake of high-density lipoprotein-associated apoprotein A-I and cholesterol esters by 16 tissues of the rat in vivo and by adrenal cells and hepatocytes in vitro. J Biol Chem 1985; 260:744-750.

(194) Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem 1959; 37:911-917.

(195) de Wael J, Raaymakers CE, Endeman HJ. Simplified quantitative determination of total fecal bile acids. Clin Chim Acta 1977; 79(2):465-470.

(196) Morris KM, Aden DP, Knowles BB, Colten HR. Complement biosynthesis by the human hepatoma-derived cell line HepG2. J Clin Invest 1982; 70(4):906-913.

(197) Stangl H, Hyatt M, Hobbs HH. Transport of lipids from high and low density lipoproteins via scavenger receptor-BI. J Biol Chem 1999; 274(46):32692-32698.

(198) Stangl H, Cao G, Wyne KL, Hobbs HH. Scavenger receptor, class B, type I-dependent stimulation of cholesterol esterification by high density lipoproteins, low density lipoproteins, and nonlipoprotein cholesterol. J Biol Chem 1998; 273(47):31002-31008.

(199) Bortnick AE, Rothblat GH, Stoudt G, Hoppe KL, Royer LJ, McNeish J, Francone OL. The correlation of ATP-binding cassette 1 mRNA levels with cholesterol efflux from various cell lines. J Biol Chem 2000; 275(37):28634-28640.

(200) Goldschmidt RC, Kimelberg HK. Protein analysis of mammalian cells in monolayer culture using the bicinchoninic assay. Anal Biochem 1989; 177(1):41-45.

(201) Ahmad S, Trepel JB, Ohno S, Suzuki K, Tsuruo T, Glazer RI. Role of protein kinase C in the modulation of multidrug resistance: expression of the atypical gamma isoform of protein kinase C does not confer increased resistance to doxorubicin. Mol Pharmacol 1992; 42(6):1004-1009.

(202) Seelig A, Landwojtowicz E. Structure-activity relationship of P-glycoprotein substrates and modifiers. Eur J Pharm Sci 2000; 12(1):31-40.

(203) Litman T, Zeuthen T, Skovsgaard T, Stein WD. Competitive, non-competitive and cooperative interactions between substrates of P-glycoprotein as measured by its ATPase activity. Biochim Biophys Acta 1997; 1361(2):169-176.

(204) Osborn MT, Berry A, Ruberu MS, Ning B, Bell LM, Chambers TC. Phorbol ester induced MDR1 expression in K562 cells occurs independently of mitogen-activated protein kinase signaling pathways. Oncogene 1999; 18(42):5756-5764.

(205) De Rijke YB, Hessels EM, van Berkel TJ. Recognition sites on rat liver cells for oxidatively modified beta-very low density lipoproteins. Arterioscler Thromb 1992; 12:41-49.

(206) De Rijke YB, Biessen EAL, Vogelezang CJM, Van Berkel TJC. Binding characteristics of scavenger receptors on liver endothelial and Kupffer cells for modified low-density lipoproteins. Biochem J 1994; 304:69-73.

(207) La Belle M, Blanche PJ, Krauss RM. Charge properties of low density lipoprotein subclasses. Journal of Lipid Research 1997; 38(4):690-700.

(208) Chappey B, Myara I, Benoit MO, Mazière C, Mazière JC, Moatti N. Characteristics of ten charge-differing subfractions isolated from human native low-density lipoproteins (LDL). No evidence of peroxidative modifications. Biochim Biophys Acta Lipids Lipid Metab 1995; 1259:261-270.

(209) Lagrost L, Florentin E, Guyard-Dangremont V, Athias A, Gandjini H, Lallemant C, Gambert P. Evidence for nonesterified fatty acids as modulators of neutral lipid transfers in normolipidemic human plasma. Arterioscler Tromb Vasc Biol 1995; 15:1388-1396.

(210) Desrumaux C, Athias A, Masson D, Gambert P, Lallemant C, Lagrost L. Influence of the electrostatic charge of lipoprotein particles on the activity of the human plasma phospholipid transfer protein. J Lipid Res 1998; 39(1):131-142.

(211) Rutenberg HL, Lacko AG, Soloff LA. Inhibition of lecithin: cholesterol acyltransferase following intravenous administration of heparin in man. Biochim Biophys Acta 1973; 326(3):419-427.

(212) Rinninger F, Pittman RC. Regulation of the selective uptake of high density lipoprotein- associated cholesteryl esters. Journal of Lipid Research 1987; 28:1313-1325.

(213) Cooper AD. Hepatic clearance of plasma chylomicron remnants. Semin Liver Dis 1992; 12(4):386-396.

(214) Darr WH, Windler EE, Stephan KU, Walli AK, Greten H. Characterization and catabolism of rat very high density lipoproteins. Journal of Lipid Research 1985; 26:672-683.

(215) Woollett LA, Spady DK. Kinetic parameters for high density lipoprotein apoprotein AI and cholesteryl ester transport in the hamster. J Clin Invest 1997; 99(7):1704-1713.

(216) Kuyvenhoven MW, West CE, van der MR, Beynen AC. Fecal steroid excretion in relation to the development of casein-induced hypercholesterolemia in rabbits. J Nutr 1986; 116(8):1395-1404.

(217) Ji Y, Wang N, Ramakrishnan R, Sehayek E, Huszar D, Breslow JL, Tall AR. Hepatic scavenger receptor BI promotes rapid clearance of high density lipoprotein free cholesterol and its transport into bile. J Biol Chem 1999; 274(47):33398-33402.

(218) Portman OW, Alexander M, O'Malley JP. Metabolism of free and esterified cholesterol and apolipoproteins of plasma low and high density lipoproteins. Biochim Biophys Acta 1980; 619(3):545-558.

(219) Schwartz CC, Vlahcevic ZR, Halloran LG, Swell L. An in vivo evaluation in man of the transfer of esterified cholesterol between lipoproteins and into the liver and bile. Biochim Biophys Acta 1981; 663(1):143-162.

(220) Schwartz CC, Berman M, Vlahcevic ZR, Halloran LG, Gregory DH, Swell L. Multicompartmental analysis of cholesterol metabolism in man. Characterization of the hepatic bile acid and biliary cholesterol precursor sites. J Clin Invest 1978; 61(2):408-423.

(221) Drevon CA, Engelhorn SC, Steinberg D. Secretion of very low density lipoproteins enriched in cholesteryl esters by cultured rat hepatocytes during simulation of intracellular cholesterol esterification. J Lipid Res 1980; 21(8):1065-1071.

(222) Rash JM, Rothblat GH, Sparks CE. Lipoprotein apolipoprotein synthesis by human hepatoma cells in culture. Biochim Biophys Acta 1981; 666(2):294-298.

(223) Hoeg JM, Demosky SJ, Jr., Edge SB, Gregg RE, Osborne JC, Jr., Brewer HB, Jr. Characterization of a human hepatic receptor for high density lipoproteins. Arteriosclerosis 1985; 5(3):228-237.

(224) Dashti N, Wolfbauer G, Alaupovic P. Binding and degradation of human high-density lipoproteins by human hepatoma cell line HepG2. Biochim Biophys Acta 1985; 833(1):100-110.

(225) Dashti N, Wolfbauer G, Koren E, Knowles B, Alaupovic P. Catabolism of human low density lipoproteins by human hepatoma cell line HepG2. Biochim Biophys Acta 1984; 794(3):373-384.

(226) Craig WY, Cooper AD. Effects of chylomicron remnants and beta-VLDL on the class and composition of newly secreted lipoproteins by HepG2 cells. J Lipid Res 1988; 29(3):299-308.

(227) Ciechanover A, Schwartz AL, Lodish HF. Sorting and recycling of cell surface receptors and endocytosed ligands: the asialoglycoprotein and transferrin receptors. J Cell Biochem 1983; 23(1-4):107-130.

(228) Ciechanover A, Schwartz AL, Dautry-Varsat A, Lodish HF. Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. Effect of lysosomotropic agents. J Biol Chem 1983; 258(16):9681-9689.

(229) Dautry-Varsat A, Ciechanover A, Lodish HF. pH and the recycling of transferrin during receptor-mediated endocytosis. Proc Natl Acad Sci U S A 1983; 80(8):2258-2262.

(230) Stangl H, Cao G, Wyne KL, Hobbs HH. Scavenger receptor, class B, type I-dependent stimulation of cholesterol esterification by high density lipoproteins, low density lipoproteins, and nonlipoprotein cholesterol. J Biol Chem 1998; 273(47):31002-31008.

(231) Romsicki Y, Sharom FJ. Phospholipid flippase activity of the reconstituted p-glycoprotein multidrug transporter. Biochemistry 2001; 40(23):6937-6947.

(232) Wigler PW. Cellular drug efflux and reversal therapy of cancer. J Bioenerg Biomembr 1996; 28(3):279-284.

(233) Wigler PW, Patterson FK. Reversal agent inhibition of the multidrug resistance pump in human leukemic lymphoblasts. Biochim Biophys Acta 1994; 1189(1):1-6.

(234) Payrastre B, Missy K, Giuriato S, Bodin S, Plantavid M, Gratacap M. Phosphoinositides: key players in cell signalling, in time and space. Cell Signal 2001; 13(6):377-387.

(235) Downes CP, Carter AN. Phosphoinositide 3-kinase: a new effector in signal transduction? Cell Signal 1991; 3(6):501-513.

(236) Haidar B, Mott S, Boucher B, Lee CY, Marcil M, Genest J, Jr. Cellular cholesterol efflux is modulated by phospholipid-derived signaling molecules in familial HDL deficiency/Tangier disease fibroblasts. J Lipid Res 2001; 42(2):249-257.

(237) Jackowski S, Rock CO. Stimulation of phosphatidylinositol 4,5-bisphosphate phospholipase C activity by phosphatidic acid. Arch Biochem Biophys 1989; 268(2):516-524.

(238) Brissette L, Falstrault L, Lafond J, Izem L. The selective uptake of the cholesteryl esters of low density lipoproteins parallels the activity of protein kinase C. Biochim Biophys Acta Lipids Lipid Metab 1996; 1301(1-2):133-140.

(239) Walter M, Reinecke H, Nofer JR, Seedorf U, Assmann G. HDL3 stimulates multiple signaling pathways in human skin fibroblasts. Arterioscler Thromb Vasc Biol 1995; 15(11):1975-1986.

(240) Nion S, Briand O, Lestavel S, Torpier G, Nazih F, Delbart C, Fruchart JC, Clavey V. High-density-lipoprotein subfraction 3 interaction with glycosylphosphatidylinositol-anchored proteins. Biochem J 1997; 328(Pt 2):415-423.

(241) Nazih-Sanderson F, Pinchon G, Nion S, Fruchart JC, Delbart C. HDL3-signalling in HepG2 cells involves glycosyl-phosphatidylinositol-anchored proteins. Biochim Biophys Acta 1997; 1346(1):45-60.

(242) Holian O, Kumar R, Attar B. Apoprotein A-1 is a cofactor independent substrate of protein kinase C. Biochem Biophys Res Commun 1991; 179(1):599-604.

(243) Folch J, Lees M, Stanley GH. A simple method for the isolation and purification and purification of total lipids from animal tissues. J Biol Chem 1957, 226, 497-509

APPENDIX

The effect of PC lipid injection on lipoprotein lipid and protein composition †

	Time h	Rabbit	CE/Protein[a] mg/mg	FC/Protein[a] mg/mg	TG/Protein[b] mg/mg	Protein[c] mg/dL
HDL						
PC	0	A	0.43 ± 0.04	0.074 ± 0.01	0.52 ± 0.01	79 ± 3
		B	0.23 ± 0.02	0.060 ± 0.01	0.12 ± 0.01	57 ± 2
	1	A	0.32 ± 0.03	0.087 ± 0.02	0.42 ± 0.01	69 ± 2
		B	0.18 ± 0.02	0.071 ± 0.01	0.12 ± 0.01	49 ± 1
	3	A	0.32 ± 0.03	0.075 ± 0.01	0.28 ± 0.01	64 ± 1
		B	0.14 ± 0.02	0.040 ± 0.01	0.16 ± 0.02	44 ± 1
	24	A	0.49 ± 0.03	0.074 ± 0.01	0.61 ± 0.01	79 ± 2
		B	0.11 ± 0.01	0.050 ± 0.01	0.21 ± 0.01	47 ± 2
LDL						
PC	0	A	1.5 ± 0.1	0.75 ± 0.05	2.6 ± 0.2	4.1 ± 0.2
		B	0.9 ± 0.1	0.25 ± 0.02	2.0 ± 0.2	1.7 ± 0.1
	1	A	1.3 ± 0.1	1.20 ± 0.08	2.3 ± 0.1	2.9 ± 0.3
		B	1.2 ± 0.1	0.67 ± 0.04	1.9 ± 0.2	1.5 ± 0.1
	3	A	1.4 ± 0.1	0.89 ± 0.06	2.6 ± 0.2	2.2 ± 0.1
		B	N.D.	0.85 ± 0.06	2.2 ± 0.3	1.4 ± 0.1
	24	A	5.8 ± 0.5	N.D.	3.7 ± 0.4	4.1 ± 0.1
		B	2.6 ± 0.2	0.75 ± 0.06	3.1 ± 0.3	3.1 ± 0.1
VLDL						
PC	0	A	0.66 ± 0.03	0.25 ± 0.02	4.7 ± 0.3	7.0 ± 0.3
		B	0.22 ± 0.02	0.15 ± 0.01	1.6 ± 0.1	5.7 ± 0.3
	1	A	0.56 ± 0.03	0.35 ± 0.01	4.9 ± 0.3	7.9 ± 0.4
		B	0.38 ± 0.05	0.21 ± 0.01	1.9 ± 0.1	5.0 ± 0.1
	3	A	0.46 ± 0.03	0.49 ± 0.03	4.6 ± 0.4	6.1 ± 0.3
		B	0.43 ± 0.08	0.17 ± 0.01	3.4 ± 0.1	5.9 ± 0.1
	24	A	0.69 ± 0.03	0.62 ± 0.04	8.0 ± 0.9	9.0 ± 0.2
		B	0.53 ± 0.05	0.37 ± 0.03	3.8 ± 0.1	7.1 ± 0.2

† HDL, LDL and VLDL at each time point from two PC-injected rabbits (A and B) were assayed for triglyceride (TG), cholesterol (FC), total cholesterol and protein. CE was calculated from the difference between FC and total cholesterol.

[a] CE/Protein TG/protein and FC/Protein values represent the mean ± S.D. in mg of lipid/mg HDL, LDL or VLDL protein of triplicate measurement.

[c] Protein values represent the mean ± S.D. in mg/dl plasma of triplicate measurements.

N.D. Not determined