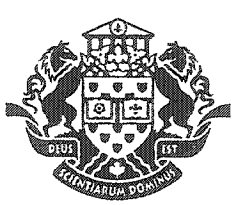

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High Density Lipoprotein Intracellular Metabolism in the Kidney

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Thesis submitted in partial fulfillment of the requirement for the degree of Masters of Science

Department of Biochemistry, Microbiology and Immunology
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Abstract

Experiments were undertaken to evaluate the factors that control the re-absorptive salvage of high density lipoproteins (HDL) in the kidney. HDL is readily taken up at the apical surface of polarized human proximal tubule epithelial cells (HKC-8). HKC-8 cells do not degrade HDL apolipoproteins, but instead transport and re-secrete the lipoprotein from the opposite, basolateral surface. Only ~10% of the HDL lipids taken up are re-secreted, while ~60% of the internalized HDL proteins are re-secreted. The composition and charge of HDL directly affects their ability to be internalized and transported through HKC-8 cells. HDL-apolipoproteins stimulate the transport of HDL components to the basolateral surface, while HDL-lipids inhibit the process. Enrichment of HDL with phosphatidylinositol (PI) increases HDL negative charge and inhibits its transport and secretion from the basolateral surface. Enrichment with phosphatidylcholine (PC) decreases HDL charge and enhances the transcytosis of HDL. These results show that HDL composition and charge regulate the re-absorptive salvage of HDL apolipoproteins in the kidney by controlling the intracellular metabolism of this lipoprotein. Our data suggest that apical to basolateral transport within proximal tubule cells could serve to recover HDL from the glomerular filtrate and return it back to the circulation. A stimulation of this process may decrease the loss of HDL from the circulation and therefore be anti-atherogenic.

Dedication

This work is dedicated to my husband.

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Abbreviations

Apo	apolipoprotein
ABCA1	adenosine tri-phosphate binding cassette protein A 1
ADPKD	autosomal dominant polycystic kidney disease
AI-BP	apoA-I binding protein
BB	brush border
BBB	blood-brain barrier
BCA	bicinchoninic acid
BHK	baby Syrian hamster kidney
BL	basal lamina
BNB	blood-nerve barrier
BSA	bovine serum albumin
Caco-2	human colon carcinoma cell line
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CHO	chinese hamster ovary
CURL	compartment of uncoupling of receptor and ligand
DAT	dense apical tubules
DG	diglyceride
DMEM	Dulbecco's Modified Eagle Medium
DPPC	dipalmitoylphosphatidylcholine
ECM	extracellular matrix
EL	endothelial lipase

ERS	electrical resistance system
FBS	fetal bovine serum
FC	free cholesterol
FcRn	Neonatal Fc receptor
HDL	high density lipoprotein
HDL-C	high density lipoprotein cholesterol
HKC-8	human kidney cells clone 8
HL	hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRP	horseradish peroxidase
HSPG	heparan sulphate proteoglycans
HUVEC	human umbilical vein endothelial cells
IDL	intermediate-density lipoprotein
IF	intrinsic factor
Ig	immunoglobulin
IRPT	immortalized rat renal proximal tubule
LCAT	lecithin-cholesterol acyl transferase
LDL	low density lipoprotein
LDLr	low density lipoprotein receptor
LLC-RK1	rabbit kidney epithelial cell line
LLC-PK1	pig renal proximal tubule epithelial cells
Lp	lipoprotein
LPL	lipoprotein lipase

LRP	low density lipoprotein receptor-related protein
mAb	monoclonal antibody
MDCK	madine-darby canine kidney
N	nucleus
OK	opossum kidney
PAGE	polyacrylamide gel electrophoresis
PAGGE	polyacrylamide gradient gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCT	proximal convoluted tubule
PEN-STREP	penicillin-streptomycin
PI	phosphatidylinositol
PKC	Protein Kinase C
PL	phospholipid
PLTP	phospholipids transfer protein
PM	plasma membrane
POPC	1-palmitoyl-2-oleoyl-phosphatidylcholine
PYY	peptide tyrosine tyrosine
RBP	retinol-binding protein
RCT	reverse cholesterol transport
rHDL	reconstituted high density lipoprotein
RPTEC	human renal proximal tubular epithelial cells
RT	room temperature

SD	**standard deviation**
SDS-PAGE	**sodium dodecyl sulfate-polyacrylamide gel electrophoresis**
SM	**sphingomyelin**
SMC	**smooth muscle cells**
SR-B1	**scavenger receptor class B1**
TCA	**trichloroacetic acid**
TD	**tangier disease**
TER	**transepithelial electrical resistance**
TG	**triacylglyceride**
TJ	**tight junction**
TNM	**tetranitromethane**
VHDL	**very high density lipoprotein**
VLDL	**very low density lipoprotein**
VLDLr	**very low density lipoprotein receptor**

Chapter 1: Introduction

The strong inverse correlation between plasma HDL concentrations, their major protein component, apolipoprotein A-I (apoA-I), and the risk of atherosclerosis has generated much interest in elucidating the mechanisms that modulate their concentrations *in vivo* (1-14). Previous studies have established that accelerated apoA-I catabolism is the main metabolic predictor of HDL levels and that plasma apoA-I levels are determined by the rate of apoA-I catabolism rather than by production (12, 15). The kidney plays a major role in HDL clearance and may be quantitatively the most important site for catabolism of HDL particles (16-22). It is believed to play an important role in the reabsorption of HDL particles and therefore an elucidation of the pathways involved is critical to understand how the levels of HDL in the bloodstream are regulated.

Atherosclerosis

Atherosclerosis is a disease of the large arteries and is the primary cause of heart disease and stroke (23, 24). In westernized societies, it is the underlying cause of about 50% of all deaths (23, 24). It is a disease characterized by the accumulation of lipids and fibrous elements in the large arteries (23-27). Atherosclerosis is a form of chronic inflammation resulting from interaction between modified lipoproteins, T cells, monocyte-derived macrophages and other arterial elements (10, 27). The early lesions of atherosclerosis typically form in branch points (bifurcations) or at curvatures in arteries, where blood flow is disturbed and turbulent (25-29). They consist of subendothelial accumulation of cholesterol-engorged macrophages, called foam cells. A triggering event for the recruitment of monocytes and lymphocytes, to the artery wall is the accumulation of minimally oxidized low density lipoprotein (LDL), which stimulates the overlying

endothelial cells to produce a number of pro-inflammatory molecules, including adhesion molecules and growth factors which results in inflammation (25-29). This leads to the unregulated uptake of oxidized or modified low density lipoprotein (LDL) particles by macrophages, which contributes significantly to monocyte recruitment and eventually foam-cell formation. The earliest detectable lesions, known as fatty streaks are characterized by the accumulation of lipid-laden macrophages (foam cells) within the intima. Fatty streaks are not clinically significant, but they are the precursors of more advanced lesions characterized by the accumulation of lipid-rich necrotic debris and smooth muscle cells (SMC). Such fibrous lesions typically have a fibrous cap consisting of SMC and extracellular matrix (ECM) that encloses a lipid-rich necrotic core (26, 27). In the final stage, atherosclerotic plaques can become increasingly complex upon calcification, ulceration at the luminal surface or haemorrhage from small vessels that grow into the lesion from the media of the blood vessel wall (27). Such plaques are unstable due to the thinning of their fibrous caps, which in turn is caused by neovascularization, and the secretion of matrix metalloproteinases by macrophages. These advanced plaques can rupture in their shoulder regions and release tissue factor into the blood stream, which leads to the initiation of coagulation, platelet recruitment and thrombus formation. Although advanced lesions can grow sufficiently large to block blood flow, the most important clinical complication is an acute coronary or carotid occlusion which is due to the formation of a thrombus or blood clot and results in a myocardial infarction or stroke (10, 25, 26).

Epidemiological studies over the past 50 years have revealed numerous risk

factors for atherosclerosis (24, 30). These can be grouped into factors with an important genetic component, and those that are largely environmental. The genetic factors include elevated levels of LDL and very low density lipoprotein (VLDL), reduced levels of HDL, elevated levels of lipoprotein(a) and homocysteine, elevated blood pressure, family history, diabetes and obesity, elevated levels of haemostatic factors, gender (male), systemic inflammation, metabolic syndrome, depression and other behavioural traits (7, 24, 26, 30, 31). The environmental factors include high-fat diet, smoking, low antioxidant levels, lack of exercise and infectious agents (7, 24, 26, 30, 31). The relative abundance of the different plasma lipoproteins appears to be of primary importance, as raised levels of LDL and low levels of HDL are a prerequisite for the development of atherosclerosis (8, 10). Although HDL metabolism is a crucial process for cholesterol homeostasis and coronary heart disease, the majority of current therapies for atherosclerosis are aimed at reducing of plasma cholesterol levels. This is often accomplished by exploiting the well-established pathways involved in the receptor-mediated metabolism of LDL. However, the mechanisms that control HDL metabolism remain mostly unknown and therefore little effort has been made to selectively target HDL levels (32, 33).

The strong inverse correlation that has been demonstrated between plasma HDL levels, their major protein component, apoA-I, and the risk of atherosclerosis has generated much interest in elucidating the mechanisms that modulate their concentrations *in vivo* (3-5, 7-14). Previous studies have established that accelerated apoA-I catabolism is the main metabolic predictor of HDL levels and that plasma apoA-I levels are determined by the rate of apoA-I catabolism rather than production (12, 15, 34-36). This

emphasizes the importance of understanding the factors and tissues that influence the catabolism of HDL in order to clarify the link between low levels of HDL and atherosclerosis.

Lipoproteins

Cholesteryl esters (CE) and triglycerides (TG) are the major neutral lipids transported through the blood stream. They are insoluble in aqueous solutions and must therefore be protected from the plasma by a coating of amphipathic molecules (37, 38). Lipoproteins are spherical macromolecular complexes that carry various lipids and proteins in plasma and provide such protection to CE and TG. They comprise a neutral core of hydrophobic molecules of CE and TG enveloped by a surface monolayer of amphipathic molecules, primarily phospholipids (PL), free cholesterol (FC), and specific proteins called apolipoproteins (37-40). The apolipoprotein component of the various lipoproteins can be broadly divided into two groups; the exchangeable (A-I, A-II, A-IV, C-I, C-II, C-III and E) and the non-exchangeable apolipoproteins (B-100 and B-48). They provide structural stability and have critical roles in regulating lipoprotein metabolism (38, 40, 41). Some apolipoproteins act as co-factors for plasma lipid-modifying enzymes. The major plasma lipoproteins can be classified into relatively distinct subclasses that can be isolated by various physical methods (see Table 1) (37).

Chylomicrons, the largest and least dense lipoproteins, contain primarily TG and are secreted by the intestine (37, 38). They function to deliver dietary TG to adipose and muscle tissue as well as dietary cholesterol to the liver. Chylomicrons leave the intestine and enter the circulation via the lymphatic system. In the capillaries of adipose and muscle tissue, fatty acids are released by the hydrolysis of TG found within

chylomicrons by lipoprotein lipase (LPL), an enzyme found on the surface of the endothelial cells of the capillaries (37, 38). Hepatic lipase (HL), a liver-localized enzyme that is similar to LPL, hydrolyzes TG from chylomicrons and VLDL remnants as well as PL from the VLDL remnants and HDL. Chylomicron remnants containing primarily cholesterol, apoE and apoB-48 are then delivered to, and taken up by, the liver through interaction with the chylomicron remnant receptor. The recognition of chylomicron remnants by the hepatic remnant receptor requires apoE (37, 38, 42).

Table 1: Physical Properties of Human Lipoproteins

Lipoprotein Class	Density (g/ml)	Molecular Weight (KDa)	Electrophoretic Mobility	Diameter (nm)	Apolipoprotein Associated
Chylomicrons	>0.93	50-100x10^3	Remains at origin	75-1200	A-I, A-II, B-48, C-I, C-II, C-III, E
VLDL	0.93-1.006	10-80x10^3	Pre-β migration	30-80	A-I, B-100, C-I, C-II, C-III, E
IDL	1.006-1.019	5-10x10^3	Slow pre-β migration	25-35	B-100, C-I, C-II, C-III, E
LDL	1.019-1.063	2.3x10^3	β migration	18-25	B-100
HDL_2	1.063-1.125	360	α migration	9-12	A-I, A-II, A-IV, E, C-I, C-II, C-III, D
HDL_3	1.125-1.21	175	α migration	5-9	A-I, A-II, A-IV, E, C-I, C-II, C-III, D
Pre-β-HDL	1.28	67	Pre-β migration	~5	A-I

Adapted from Havel, R.J. and Kane, J.P. 2001. Introduction: Structure and Metabolism of Plasma Lipoproteins. In Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D., editors. *The Metabolic and Molecular Basis of Inherited Disease*, McGraw-Hill Companies, Inc., New York

The dietary intake of both fat and carbohydrate in excess of the needs of the body leads to their conversion into TG in the liver (37, 38). These TG are packaged into VLDL

and released into the circulation for delivery to the various tissues (primarily muscle and adipose tissue) for storage or production of energy through oxidation. In addition to TG, VLDL contains some cholesterol and CE and the apolipoproteins, apoB-100, apoC-I, apoC-II, apoC-III and apoE. Like nascent chylomicrons, newly released VLDL acquires apoC and apoE from circulating HDL (37, 38). Fatty acids are released for use by adipose and muscle tissues in the same way as for chylomicrons, through the action of LPL. As LPL and HL converts VLDL to intermediate density lipoproteins (IDL), also termed VLDL remnants, the apoC are transferred to HDL. The predominant remaining proteins are apoB-100 and apoE. Further loss of TG converts IDL to LDL. ApoE is also lost at this stage. The fate of IDL is either conversion to LDL or direct uptake by the liver. Conversion of IDL to LDL occurs as more TG are removed and CE is received from HDL through the action of cholesterol ester transfer protein (CETP). The liver takes up IDL after they have interacted with the LDL receptor (LDLr) to form a complex, which is endocytosed into the cell. The mammalian LDLr family comprises a considerable number of receptors characterized by distinct functional domains (43). Besides LDLr, other members of this family include the LDLr-related protein (LRP), megalin and the VLDL receptor (VLDLr). These receptors are expressed in various tissues including intestine, lung, brain, muscle, and skin, with the highest expression in the liver (43, 44).

The cellular requirement for cholesterol as a membrane component is satisfied in one of two ways: either it is synthesized *de novo* within the cell, or it is supplied from extracellular sources, namely, HDL and LDL (38). Cholesterol synthesized by the liver can also be transported to extra-hepatic tissues packaged in VLDL. In the circulation VLDL are converted to LDL through the action of LPL and HL. LDL are taken up by

cells via LDL receptor-mediated endocytosis. The hepatic LDLr-mediated uptake of LDL is an important process for the removal of cholesterol from the circulation and involves the interaction of the LDLr with apoB-100, the only apolipoprotein on LDL (38, 45).

HDL are the smallest (7.0-12 nm diameter) and the most dense ($1.063 < d < 1.25$ g/ml) of the plasma lipoproteins (1). HDL are a diverse population of particles both in structure and route of formation. HDL FC is esterified through the action of the HDL-associated enzyme, lecithin:cholesterol acyltransferase (LCAT). The CE of HDL can also be transferred to VLDL and LDL through the action of the HDL-associated enzyme, CETP. This has the added effect of allowing the excess cellular cholesterol to be returned to the liver through the LDL-receptor pathway as well as by unique HDL-receptor pathways. CE in HDL and LDL can be selectively taken-up by the scavenger receptor class BI (SR-BI) into hepatocytes and steroid hormone-producing cells without internalizing HDL proteins (7, 8, 38, 46, 47).

HDL mediates the transport of cholesterol from non-hepatic cells and its subsequent delivery to the liver and steroidogenic organs, where it is used for the synthesis of lipoproteins, bile acids, Vitamin D, and steroid hormones. Thus, there is considerable interest to develop anti-atherogenic strategies involving enhanced cholesterol efflux and reverse cholesterol transport (RCT) (8, 37, 38, 42).

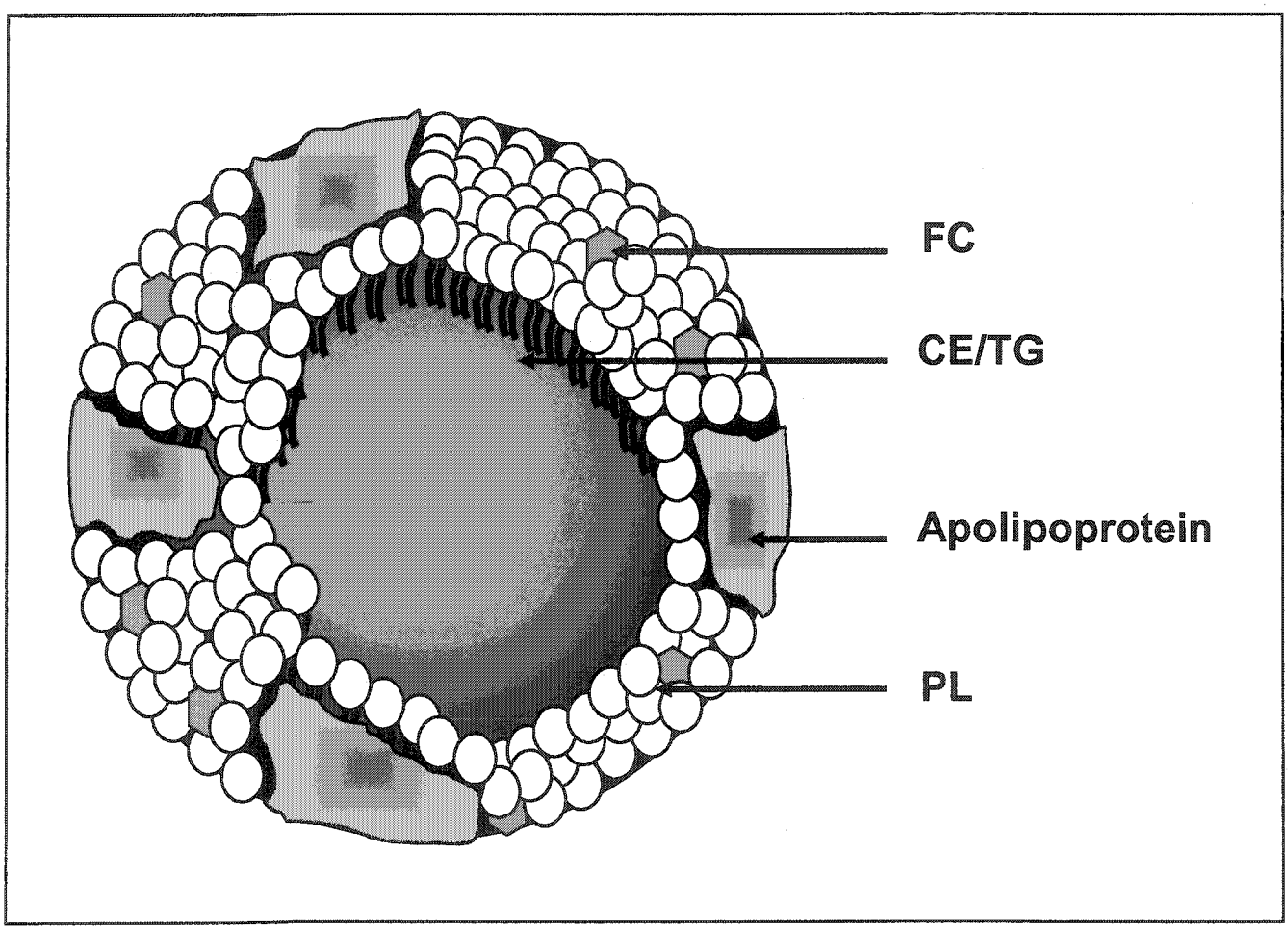
HDL Metabolism

HDL Maturation

HDL is a dense, protein rich particle, with a small apolar core surrounded by a surface layer of apolipoproteins and amphiphilic lipid molecules (Fig. 1) (37). HDL encompasses a heterogeneous class of lipoproteins, where differences in the content of

Figure 1 : **Schematic Representation of HDL**

The HDL is a dense, protein rich particle with a small apolar core comprised mainly of CE and TG, surrounded by a surface layer of apolipoproteins (mainly apoA-I), amphiphilic PL and FC. HDL circulates in the bloodstream, extracting cholesterol from body tissues and transporting it to the liver for excretion or recycling.


FC
CE/TG
Apolipoprotein
PL

lipids, apolipoproteins, enzymes, and lipid transfer proteins result in the formation of various HDL subclasses. These classes are characterized by differences in shape, density, size, charge, and composition (Table 1, 2). The major protein present in HDL is apoA-I, comprising about 70% to 80% of the protein mass. It is synthesized in both the liver and small intestine. ApoA-I is believed to be responsible for the anti-atherogenic and anti-thrombogenic properties of HDL (37, 41, 48-50). HDL also acts as a circulating reservoir of apoC-I, apoC-II and apoE and can contain apoA-II and apoA-IV. HDL exists as three major subclasses, HDL_2, the largest and least dense HDL particle, HDL_3 and VHDL, the smallest and most dense particles. HDL_2 and HDL_3 are further subdivided by size into subclasses, HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b} and HDL_{3c} (1, 51).

Table 2: Composition of HDL Subclasses

HDL subclass	Composition (% w/w)				
	Protein	PL	FC	CE	TG
Pre-β-HDL	70	25	5	nd	nd
HDL_3	55	25	3	16	1
HDL_2	43	30	5	20	2

PL, phospholipid; FC, free cholesterol; CE, cholesteryl ester; TG, triacylglycerol; nd, not detectable.

Adapted from Fielding, P.E. and C.J. Fielding, 1996. *Biochemistry of Lipids, Lipoproteins and Membranes, In* Vance, D.E. and J.E. Vance, eds., New York, New York: Elsevier

Nascent HDL precursors, containing apoA-I and PL are synthesized by the liver and intestine. They have a characteristic pre-β electrophoretic migration on agarose that distinguishes them from the bulk of α-migrating HDL (38, 52). These lipid-poor HDL precursors are produced either as nascent HDL by hepatocytes and the intestinal mucosa,

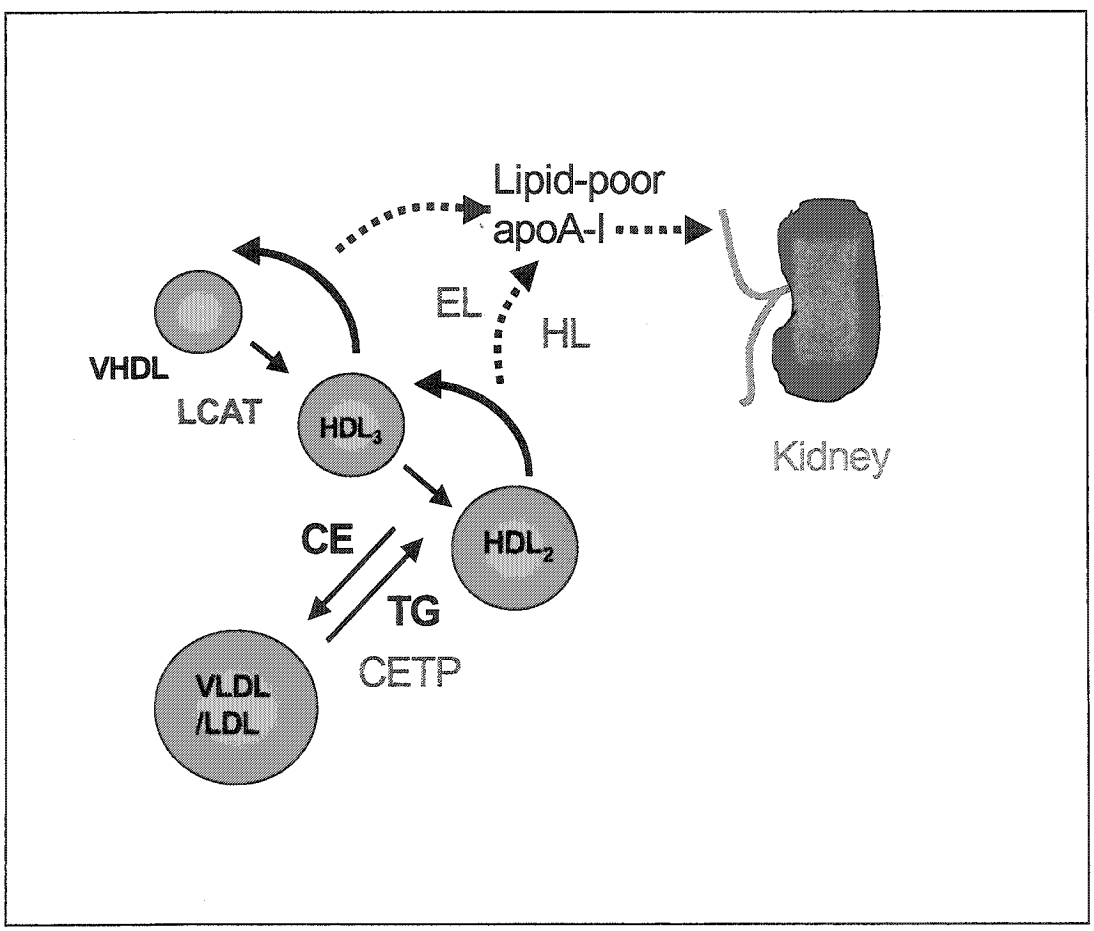
or they dissociate from chylomicrons and VLDL during LPL-mediated hydrolysis of TG. LPL promotes the transfer of lipids and apolipoproteins to HDL (2). Nascent HDL can also be generated by the interconversion of HDL_2 and HDL_3 by CETP, phospholipids transfer protein (PLTP), and HL (7, 8). Nascent pre-β-HDL acquire PL and unesterified cholesterol from peripheral cells through the action of an adenosine tri-phosphate binding cassette protein A1 (ABCA1)-mediated efflux. Progressive lipidation converts these particles into spherical α-migrating HDL. A deficiency of ABCA1 in patients with Tangier disease (TD) or in knockout mice causes a defect in cellular lipid efflux and results in the absence of lipid-rich α-migrating HDL in plasma. TD patients appear to have unique defects in the gene encoding ABCA1 (53-57). The initial products of this ABCA1-mediated lipid efflux are small HDL_3 particles. Esterification of FC by LCAT and remodeling with other HDL constituents by PLTP result in the formation of larger HDL_2 particles. The activity of LCAT requires interaction with apoA-I, which is found on the surface of HDL. Continued adsorption of FC followed by LCAT-driven esterification enables HDL_3 to increase in size and accommodate apoC-II and apoC-III derived from apoB-containing lipoproteins. ApoE can also be transfered to HDL at this time (1, 8, 9, 58-60). The result of these processes is the formation of a mature HDL_2 (Fig. 2).

HDL Catabolism

The lipids and proteins of mature HDL particles can be removed from the circulation by indirect pathways that involve the action of CETP, HL, and endothelial lipase (EL), as well as direct pathways, which involve the selective lipid uptake by SR-BI and the holoparticle uptake by unique receptors, such as SR-BI, cubilin and other potential HDL receptors (8, 61).

Figure 2: Brief Overview of HDL Metabolism

Mature HDL particles that are generated from lipid-poor nascent HDL precursors are synthesized by the liver and intestine. Nascent HDL particles acquire excess FC and PL from peripheral cells through a lipid efflux pathway mediated by ABCA1. The surface FC is esterified by the enzyme LCAT to form CE, which moves to the core of the HDL. Through the action of LCAT, HDL is progressively enlarged, taking the form of HDL_3 and eventually HDL_2 particles. Through the action of CETP, CE can be transferred from HDL to apoB-containing lipoproteins in exchange for TG, or selectively removed by the SR-B1 receptor in the liver. Hydrolysis of TG-rich lipoproteins leads to the transfer of lipids and apolipoproteins to HDL. Mature HDL_2 is progressively degraded into HDL_3 and nascent HDL. Nascent HDL and lipid-poor apoA-I can be metabolized by the kidneys and undergo a glomerular filtration and tubular reabsorption.


Lipid-poor
apoA-I
EL
HL
VHDL
LCAT
HDL3
Kidney
CE
HDL2
TG
CETP
VLDL
/LDL

The concerted action of CETP, HL and EL converts larger HDL_2 into smaller HDL_3 and releases lipid-poor apoA-I and pre-β-HDL particles (2). Increased transfer of CE from HDL to apolipoprotein B-containing lipoproteins via CETP is probably a major factor in the low HDL cholesterol levels associated with hypertriglyceridemia and type 2 diabetes (8, 62, 63). Reduced HDL catabolism and elevated HDL-C levels in individuals with CETP deficiency have been observed (8, 62, 63). Mice naturally lack CETP, and transgenic expression of CETP in mice reduces plasma HDL-C levels (64). HL hydrolyzes PL and TG in all lipoprotein classes (65). Since ApoA-II inhibits HL, HDL devoid of apoA-II are the preferred substrate for HL (8, 9, 66-68). Genetic HL deficiency in humans is associated with modestly elevated HDL cholesterol levels and larger HDL particles (69, 70). EL hydrolyzes HDL-PL, generating lipid-depleted HDL. EL is a member of the same gene family as LPL and HL (71, 72). Although EL has some TG lipase activity, it has has substantially more phospholipase activity than LPL or HL (73). Furthermore, it is more effective at hydrolyzing lipids in HDL than LPL or HL (74). Overexpression of human EL in the livers of mice with a recombinant adenoviral vector caused markedly reduced plasma concentrations of HDL-C and apoA-I (71). On the other hand, inhibition of EL in mice results in increased HDL-C and apoA-I levels (61). The newly generated lipid-free apoA-I and pre-β-HDL by EL, HL and CETP activity may then be available for further sterol removal from peripheral tissues through the RCT pathway (8).

ApoA-I and HDL play an important role in cholesterol homeostasis by promoting cholesterol efflux from peripheral cells and returning it to the liver for excretion into the bile, a process known as RCT (2). This cholesterol can also be transferred to newly

secreted plasma lipoproteins (8, 59, 66, 75). SR-BI mediates the selective uptake of CE from HDL into hepatocytes and steroid hormone–producing cells and may therefore be important in the regulation of RCT (76-83). By expression cloning, Acton *et al.* isolated the cDNA for a new member of the CD36 family of membrane proteins, named SR-BI, while investigating the physiological properties of CD36 in a variant Chinese Hamster Ovary (CHO) cell line using acetylated LDL as a ligand (84-86). In contrast to holoparticle receptors (eg, members of the LDL receptor gene family or class A scavenger receptors), SR-BI is predominantly found in caveolae and targets internalized lipids to non-endosomal and non-lysosomal compartments. Epitopes recognized by SR-BI appear to include PL and apolipoproteins. In addition, selective uptake by SR-BI may depend on the presence of co-factors like HL (8, 9, 66-68). Studies show that SR-BI knockout mice have increased HDL cholesterol levels (76, 87), and hepatic overexpression of SR-BI in mice results in markedly reduced HDL-C levels (20, 88, 89). SR-BI is also expressed in macrophages and may contribute to cholesterol efflux from these cells under certain conditions (90). The net movement of cholesterol into or out of the cell may depend on the relative activities of extracellular lipid transfer enzymes (LCAT, PLTP, and CETP) and intracellular enzymes, which metabolize cholesterol to CE, bile acids, lipoproteins, or steroid hormones (8, 9, 66-68).

The removal of the protein constituents of HDL from the circulation is less well understood than the removal of HDL-associated lipids. Potential mechanisms for the catabolism of HDL and lipid-poor apolipoproteins are endocytosis into liver and kidney cells as well as into placenta and yolk sac during pregnancy (8, 91-95). The liver and kidney are sites of preferential uptake and degradation of apoA-I (16-22, 96-98). Within

the kidney, the proximal tubule is the major site for reabsorption of filtered lipid-free apoA-I and small HDL particles with a size of <8 nm (96, 99-104). The receptor for the intrinsic factor/vitamin B12, cubilin, has been identified as an HDL/apoA-I binding site in epithelial cells of the proximal tubule of the kidney and yolk sac. Cubilin has been suggested to mediate the uptake of apoA-I into proximal tubule cells. However, cubilin has no transmembrane domain and can therefore not mediate the internalization of its ligands without co-receptors (92). Megalin, a member of the LDL receptor gene family, has been suggested to play this role in the kidney and the yolk sac (8). When Glass *et al.* studied the tissue sites of degradation of apolipoprotein A-I, they found high rates of apoA-I renal uptake (85). Their studies also suggested that the high uptake by the kidney was due to glomerular filtration, but that a tubular reabsorption of free apoA-I may prevent losses in the urine (85). The importance of kidney in apoA-I catabolism was further highlighted by observations from studies of the metabolic turnover of serum HDL apolipoproteins in rats (95). The study indicated that partial hepatectomy had no effect on the fractional turnover rate of HDL apolipoproteins and that HDL lipoproteins are catabolised in the kidneys and liver (95). Furthermore, Braschi and co-workers re-evaluated the kinetics of unmodified apoA-I and a reconstituted HDL particle (LpA-I) in rabbits after a unilateral nephrectomy (22). Removal of one kidney was associated with a 40-50% reduction in creatinine clearance rates and a 34% decrease in the clearance rate of unmodified apoA-I and reconstituted rHDL particles. In contrast, the clearance of ^{125}I-labeled molecules was much less affected by the removal of a kidney. The data shows that the kidneys are responsible for most (70%) of the catabolism of apoA-I and HDL *in vivo*, while ^{125}I-labeled apoA-I and HDL are rapidly catabolized by different

tissues (22). Therefore, degradation of HDL apolipoproteins in the kidney appears to be the rate-limiting step in the overall catabolism of HDL apolipoprotein.

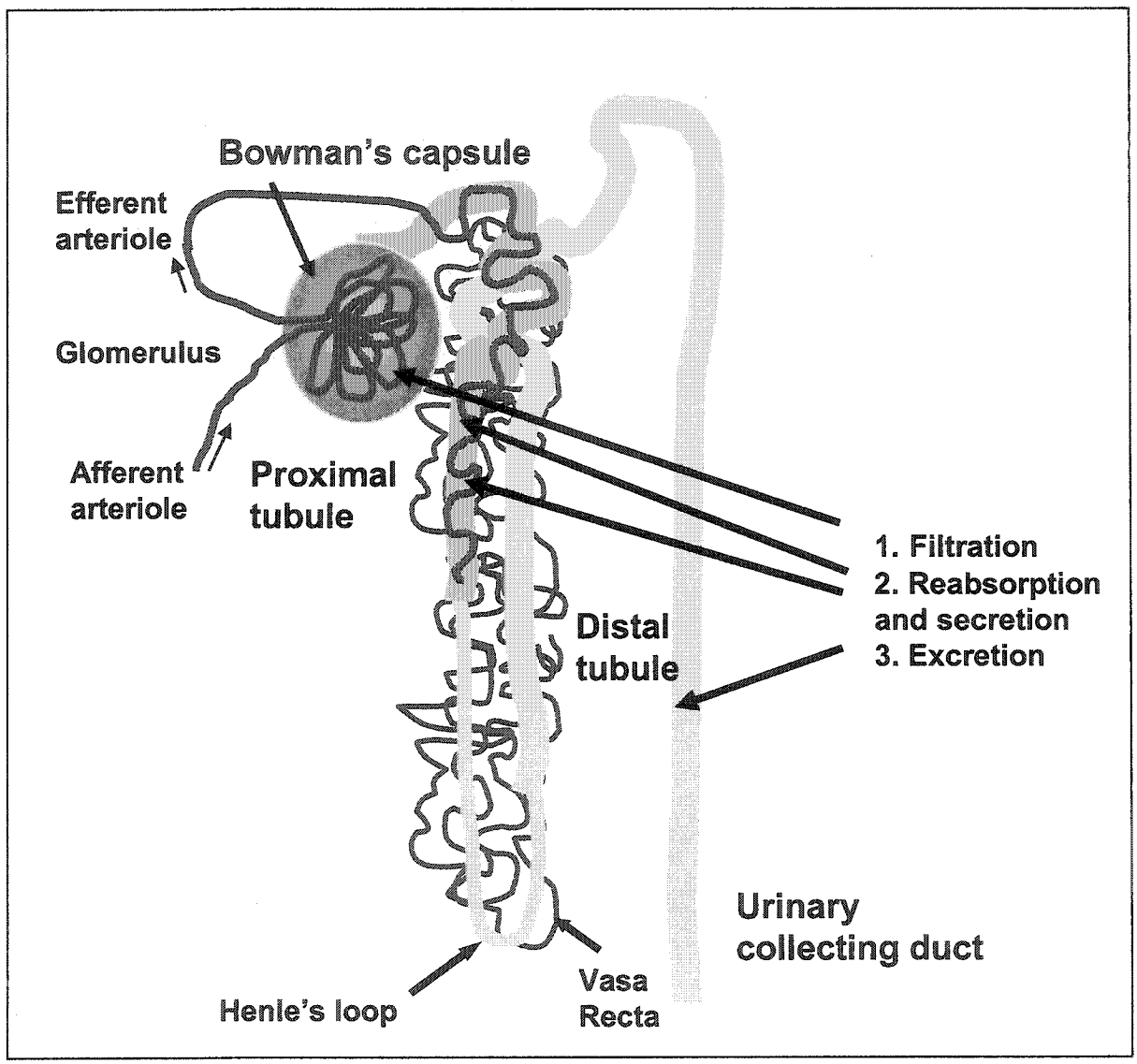
Renal Physiology

The Nephron

The basic functional unit of the kidney is the nephron (Gr. *nephros*, kidney). The kidney can be divided into an outer cortex and an inner medulla (105). Each kidney is composed of 1-4 million nephrons within its outer cortex. Each nephron consists of the renal corpuscle (glomerulus and Bowman's capsule), the proximal convoluted tubule (PCT), the thin and thick limbs of Henle's loop and the distal convoluted tubule (Fig. 3). The collecting tubules and ducts, whose embryonic origin differs from that of the nephron, collect the urine produced by nephrons and conduct it to the renal pelvis (105). Filtration takes place in the glomerulus, where an ultrafiltrate of blood plasma is formed. Each renal corpuscle consists of a bed of capillaries, the glomerulus, surrounded by a double-walled epithelial capsule called the Bowman's capsule. The internal layer (visceral layer) of the capsule envelops the capillaries of the glomerulus and the outer parietal layer of the Bowman's capsule forms the outer limit of the renal corpuscle (105). Between the two layers is the urinary space, which receives the fluid filtered through the capillary wall and the visceral layer. Each renal corpuscle has a vascular pole, where the afferent arteriole enters, the efferent arteriole exits and the PCT begins. The incoming afferent arteriole divides into two to five primary branches, which then subdivide into capillaries and form the renal glomerulus. The efferent arteriole arises from the convergence of capillaries arising from multiple lobules within the glomerulus (105, 106). The efferent arteriole branches into the peritubular capillary network that nourishes

Figure 3: Schematic Representation of a Nephron

The afferent arteriole brings the systemic blood into the glomerulus and forms the glomerular capillary bed, where the blood is filtered. From the glomerular capillaries the blood exits the glomerulus through the efferent arteriole to nourish the tubular network of the nephron and to eventually leave the kidney via the venous system. The filtration of the blood plasma components across the filtration barrier between the capillaries and the urinary space of the glomerulus is complex and is governed by the size, charge and shape-selective properties of the filtration barrier. The collected plasma ultrafiltrate exits the lumen of the Bowman's capsule to enter the proximal tubule, the Henle's loop, the distal tubule and the collecting duct, which are involved in the reabsorption, processing and secretion of useful components of the ultrafiltrate. The collected urine is then excreted from the kidneys via the ureter.


Bowman's capsule
Efferent arteriole
Glomerulus
Afferent arteriole
Proximal tubule
1. Filtration
2. Reabsorption and secretion
3. Excretion
Distal tubule
Urinary collecting duct
Henle's loop
Vasa Recta

the proximal and distal tubules and carries away larger and negatively charged plasma components that are unable to penetrate the glomerular filtration barrier. Some of these components can be reabsorbed from the peritubular circulation if capable of transversing across the tubular basement membrane to encounter the basolateral cell surface of the renal proximal tubule cells (PT cells) (105).

The glomerulus is a selective filtration membrane. The glomerular filtrate is formed in response to the hydrostatic pressure of blood (40-60 mm Hg), which is opposed by the osmotic pressure of plasma colloids (20 mm Hg), and the hydrostatic pressure of the fluids in Bowman's capsule (10 mm Hg). This results in a net filtration pressure of 15 mm Hg at the afferent end of the glomerular capillaries (105). Endothelial cells lining the glomerular capillaries are fenestrated with numerous pores 70-90 nm in diameter and they form the initial barrier to the passage of plasma constituents from the capillary lumen of the glomerulus to the urinary space of the Bowman's capsule. The glomerular basement membrane contains fibronectin, collagen IV and laminin in a matrix containing the negatively charged heparan sulphate proteoglycans (HSPG). Thus the glomerular basement membrane forms the main filtration barrier between the blood in the capillaries and the urinary space, with both physical and charge selective barrier properties (107, 108). The factors that determine which molecules are filtered are molecular size, electrical charge, protein binding, shape and rigidity. The filtration barrier is effective in preventing the entry of the majority of particles greater than 10 nm in diameter. Since the filtration barrier has a net negative charge, the movement of large negatively charged molecules is restricted more than molecules with a positive or neutral charge. While spherical molecules with a mean radius greater than 50 Å, will hardly

penetrate the glomerular filtration barrier, molecules that display asphericity under shear could present smaller effective radii to the filtration barrier, which enables their filtration (109, 110). Several structural studies have shown that HDL may also exhibit an elongated, flexible configuration (111-114). The special filtering characteristics of the glomerulus coupled with the unique renal circulation allow for effective glomerular filtration to occur (105).

The primary function of the kidney is to maintain body volume and electrolyte balance, while removing nitrogenous and other waste products from the blood, is dependent upon the selective reabsorption of water, ions and macromolecules by proximal and distal tubule cells (115-117). The plasma ultrafiltrate that forms in the glomerulus passes into the PCT, where the process of absorption and excretion begins. The PCT has an initial convoluted section (pars convuluta) that begins at the urinary pole of the glomerulus and a straight section (pars recta), which extends into the medulla. The PCT is lined with simple cuboidal or columnar epithelium. The epithelial cells have an acidophilic cytoplasm that results from the presence of numerous elongated mitochondria. The cells have three to five spherical nuclei, usually located in the center of the cell. They also contain large quantities of smooth and rough endoplasmic reticulum, free ribosomes and have a well developed Golgi apparatus located above the nuclei (107). PCT cells have a polarized surface membrane. A distinctive apical membrane, which consists of microvilli separated from a basolateral membrane domain by cellular junctional complexes, structurally characterizes proximal tubule cells (118-120). While the apical side of the PCT faces the tubular lumen, the basolateral regions rest on the tubular basement membrane, which separates the cells from the

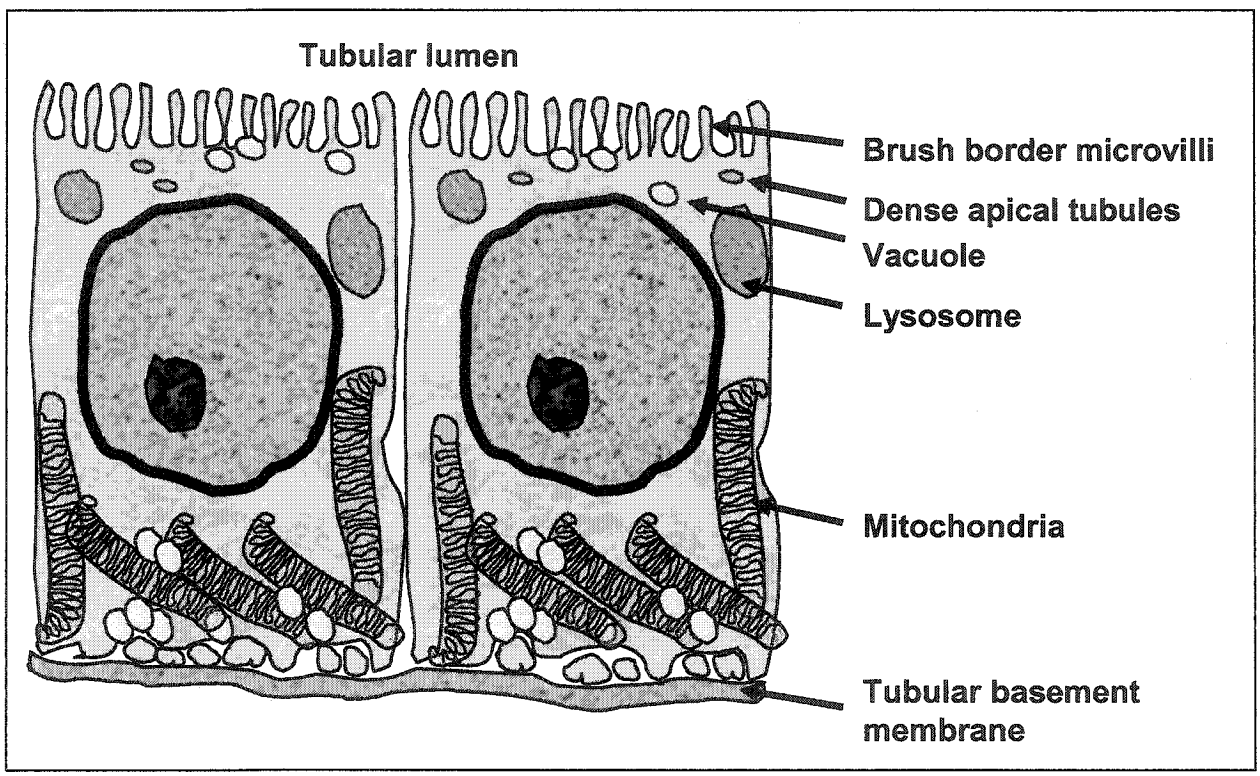
underlying peritubular capillaries. The microvilli increase the apical membrane surface by 40-fold, greatly increasing the absorption capabilities of these cells. The membrane that surrounds the microvilli has a unique composition of lipids and peripheral membrane proteins for maximizing peptide and glucose absorption. The apical cytoplasm of these cells has numerous canaliculi between the bases of the microvilli which are involved in rounding up pinocytotic vesicles and fusing them with lysosomes. They represent the initial elements of the well developed endocytic apparatus of the PCT (105, 117, 121). The lateral and basal portions of the cells have abundant membrane invaginations and lateral interdigitations with neighboring cells. The basolateral membrane also contains sodium pumps for active ion transport. The integrity of these polarized cellular membranes and structures is thus critical to the normal absorptive and secretory functions of these cells. While intermediate junctions and desmosomes provide a mechanical link between adjacent cells, tight junctions are responsible for intercellular sealing and regulation of paracellular transport of specific solutes, ions and water (122, 123). For a schematic depiction of PCT cells see Figure 4.

Protein Handling in the Proximal Tubule

The glomerular filterate formed in the renal corpuscle passes into the proximal convoluted tubule, where the macromolecular components of the plasma ultrafilterate are reabsorbed by the cells of the PCT (94). The PCT absorbs all the glucose and amino acids and about 85% of the sodium chloride and water contained in the filtrate in addition to proteins, vitamins, phosphate and calcium (124). The reabsorption of proteins occurs largely by adsorptive endocytosis. The endocytic apparatus consists of apical clathrin coated pits/endocytic invaginations, small (<0.5 μm) coated and non-coated early

Figure 4: Schematic Representation of Proximal Tubule Cells

The apical surface of the cuboidal epithelial PCT cells faces the renal tubular lumen. They have an extensive microvillar brush border which greatly increases the absorptive surface area of the apical membrane. The PCT cells are enriched in mitochondria and are multinucleated. They also possess a well-developed endocytic apparatus comprising of clathrin coated pits, vacuoles, dense apical bodies and lysosomes. The basolateral surfaces of the PCT cells are characterized by long interdigitating membrane processes, which contact the tubular basement membrane. The basement membrane represents a selective barrier that separates the PCT cells from the peritubular capillaries. The adjacent PCT cells are separated by intercellular spaces, with junctional areas where the paracellular diffusion is tightly regulated via structures such as tight junctions.


Tubular lumen
Brush border microvilli
Dense apical tubules
Vacuole
Lysosome
Mitochondria
Tubular basement membrane

endosomes, large (>0.5 μm) non-coated endosomes which can be subdivided into endosomes with (early) and without (late) an internal coat, dense apical tubules (DAT) (0.07-0.09 μm) free in the apical cytoplasm connected to endosomes, the lysosomes and small tubular structures (<0.5 μm) smaller and different from DAT (125). The clathrin coated pits are located at the base of the very well-developed apical brush border (126). Such pits eventually give rise to clathrin-coated endocytic vesicles, which will transport their cargo into the cell interior (127). Several peptidases and hydrolases have been localized to the microvilli of the PCT brush border by different methods, which constitute a pathway for the degradation of low molecular weight peptides (128, 129). However, endocytotic uptake and intracellular transport at the electron microscope level have been studied for a variety of larger peptides and proteins in the proximal tubule (130). The initial event in the adsorptive endocytosis process is binding of the proteins to sites located at the luminal plasma membrane, either directly in the invaginations or at the brush border from where they may migrate laterally in the plane of the membrane into invaginations. Several key receptors appear to be involved in the endocytic reabsorption of components of the ultrafiltrate. Specific receptors like the folate receptor, IGF-II/Man-6-P receptor, and gp280/IFR, identical to the intrinsic factor receptor also play a role in the apical endocytic pathway of renal proximal tubules (130). Research has suggested that cubilin and megalin are probably the most important receptors in the reabsorption process in the proximal tubule mediating endocytosis of a large variety of ligands (91, 92, 98, 131-133). Megalin binds cubilin and co-localizes with it in apical endocytic invaginations and endosomes of PCT cells (93, 94, 132, 134-138). Recent findings have also shown the existence of a novel apoA-I binding protein, AI-BP (139). The study

showed that stimulation of cells derived from the kidney proximal tubules with apoA-I or HDL induces a concentration-dependent secretion of AI-BP, indicating an important role for AI-BP in the renal tubular degradation or reabsorption of apoA-I (139). After binding of protein to the extracellular coat, the invaginations are pinched off to form larger endocytic vacuoles (late endosomes) still provided with an internal coat but generally without a cytoplasmic coat. Dense apical tubules, often seen connected to endocytic vacuoles are thought to be responsible for recycling membrane proteins between the endocytic vacuoles and the apical plasma membrane. The protein ligands are transferred to lysosomes from endocytic vacuoles for degradation (124, 140, 141). While a majority of ligands that undergo endocytosis are lysosomally degraded, a small amount can escape degradation (267). It has been suggested that internalized protein may be transported to subcellular compartments other than lysosomes (124). For example, cationic peroxidases have been localized in small amounts in the Golgi apparatus after internalization in the proximal tubule (142). Studies have shown that proteins such as peroxidase (143), lysozyme (144), albumin (145), catalase (146) and very large molecules like ferritin (143) can penetrate or diffuse retrograde from the peritubular space through the tubular basement membrane into the intercellular space. Receptors for insulin, parathyroid hormone, epidermal growth factor, growth hormone (147), HDL (103) and other macromolecules have been demonstrated at the basolateral membrane of the PCT cell.

The existence of transtubular transport of proteins from the lumen of the proximal tubule to the peritubular space has received much discussion about the extent and role of either transcellular or paracellular transport (124, 148). Bulger and Trump suggested that endocytic vacuoles or deep plasma membrane invaginations could empty their contents

into the intercellular space thereby allowing protein to pass the tubule wall undegraded (149). Experiments with isolated, horseradish peroxidase (HRP) or cationized ferritin-perfused rabbit kidneys showed that HRP- or ferritin-filled vacuoles were transported from the apical to the basolateral side of the cells, strongly suggesting transcellular transport (150). Goligorsky and Hruska demonstrated that cultured PT cells can facilitate significant transcytosis of the internalized ligands (148).

Cell Biology of Proximal Tubule Epithelial Cells

Cell Polarization

Polarity is a fundamental property of cells in living organisms that is required for embryonic development as well as adult physiology (151-154). Epithelial cells, with their morphologically distinct apical and basal regions separated by cell junctions, have long been a favorite system for the study of metazoan cell polarity. A primary function of epithelial cells is to provide a boundary between different extracellular compartments. As a consequence, these cells exhibit cell-surface polarity, which means that their plasma membrane is divided into specialized regions that are exposed to different environments and which have a characteristic protein and lipid composition. The basolateral plasma membrane domain faces the blood circulation and adjacent cells, whereas the apical domain is in contact with the external environment, such as the bile canaliculus in hepatocytes or the lumen in renal and intestinal epithelial cells (155-166). These domains are separated by tight junctions (TJ) to prevent intermixing of membrane components. Organisms contain other cells, however, that lack morphological asymmetry but are nonetheless highly polarized. As a functional adaptation for the location between blood and tissues, the endothelial cells are also polarized, exhibiting a luminal and abluminal

front (167, 168). Biochemically differentiated microdomains that correspond to structures involved in endocytosis and transcytosis are generated on the endothelial cell surface due to difference in surface charge (167). The preferential distribution of specific receptors on the luminal or abluminal plasmalemma, play a critical role in the uptake, intracellular routing, and direction of transport of their respective ligands. Disturbances of cell polarity may contribute to the pathogenesis of disease states, including ischemic and radiocontrast-induced acute renal failure and carcinomas (167). These examples demonstrate the biological and potentially clinical relevance of cell polarity in the system (167). To generate and maintain this structural polarity, cells need mechanisms to specifically target newly synthesized proteins and lipids to the correct surface (162). Revealing the mechanisms and regulation of these polarized transport processes is a major challenge.

To obtain a detailed understanding of polarized membrane trafficking, it is important to reveal how transport of proteins and lipids are regulated (155). Well characterized polarized *in vitro* model systems would simulate the *in vivo* condition, which can be manipulated to measure the effects of different variables (e.g. temperatures, pharmacological agents) to explore the molecular mechanism of polarized membrane trafficking pathways (169). Although the mechanisms for polarized protein targeting are basically similar in all polarized cells, and are probably even preserved in cells that do not display cell-surface polarity, the relative importance of the targeting pathways differs strongly and depends on the cell type (155, 169). A widely adopted model system is the Madin–Darby canine kidney (MDCK) cell line, which, when grown on permeable filters, forms a well-polarized monolayer that can be manipulated from both the apical and the

basolateral side (170-175). A human colon carcinoma cell line (Caco-2) is used similarly as a model for polarized intestinal cells (78, 176, 177). Studies on the mechanisms of polarized transport in hepatic cells have been hampered by the lack of suitable model systems. Freshly isolated hepatocytes can be used as an *in vitro* polarized cell-culture model. Many studies show that hepatocytes may be isolated as pairs, or 'couplets', by limited collagenase perfusion (178-180). In hepatocyte couplets, canalicular bile secretion and apical lipid transport (181-183) have been successfully studied. Some cell lines like the WIF-B cell line, a rat hepatoma hybrid cell line, have been reported to show hepatocyte-like polarity (156, 184-188). Human umbilical vein endothelial cells (HUVEC) have also been cultured on a two-chamber culture model in a polarized pattern (155, 169, 189).

The kidney is believed to play an important role in the re-absorption of HDL particles and therefore an optimized differentiated human renal epithelial cell line with extended *in vitro* growth potential would provide an alternative model system to primary culture or other available non-human mammalian kidney cell lines (190). For this purpose, human renal tubule epithelial cells were isolated from normal kidney cortex and exposed in culture to a hybrid immortalizing virus, adenovirus 12-SV40. This immortalized human renal PT epithelial cell line, the HKC-8, expresses differentiated features of proximal tubular cells and is compared to, and in some cases superior to, established cell lines (LLC-RK1, OK, HK-2) and to human PT cells in prolonged primary culture. The cell line has been shown to maintain normal expression of tubule markers and biochemical properties and to display normal epithelial monolayer morphology with a well developed brush border for prolonged periods (190). Polarized HKC cells, MDCK

cells and ADPKD (autosomal dominant polycystic kidney disease) cells have been used in different studies (170, 191-198). In recent studies, Dai *et al.* have used HKC cells to study the mechanisms for tubular cell loss (195, 199, 200). Bland *et al.* used HKC-8 cells to study the regulation of Vitamin D regulation by calcium. Polarized HKC-8 cells are a good model to explore the molecular mechanism of polarized membrane trafficking pathways in the human proximal tubules.

Polarized Membrane Trafficking Pathways

The existence of extensive membrane traffic pathways in eukaryotic cells is now well recognized (155, 201). Transcytosis, paracellular and transcellular pathways are the three major membrane trafficking pathways used to transport cargo from one side of the cell to the other. Together, these three processes contribute to the success of multicellular organisms (155, 161, 162, 169, 202, 203). However, the relative importance of each targeting pathway is dependent on the cell type (155, 204). In endothelial cells, the permeant molecules can be either internalized by endocytosis or may be translocated across the cell to the interstitial fluid by transcytosis. Endocytosis is a property of eukaryotic cells whereby membrane proteins and lipids, extracellular ligands and soluble molecules from the cell surface are taken up in membrane bound vesicles (205-207). Endocytosis and transcytosis are interconnected pathways in a network of membrane traffic (162, 203, 208). In comparison, epithelial transport occurs through both transcellular pathways, via caveolae and channels, and paracellular via intercellular junctions.

Transcellular transport, the movement of ions and small molecules through a cell, is accomplished by the differential distribution of membrane transporters/carriers on

opposite sides of a cell whereas paracellular transport, the movement between adjacent cells, is accomplished by the regulation of tight junction permeability. Transcellular transport is directional, energy dependent, and governed by the cell-specific profile of transporters and channels positioned on the apical and basolateral cell membranes. Paracellular transport on the other hand, is passive and results from diffusion, electrodiffusion, or osmosis down the gradients created by transcellular mechanisms. The paracellular route does not show directional discrimination; however, it varies enormously among epithelia in terms of electrical resistance and shows small differences in ionic selectivities. Thus the paracellular pathway complements transcellular mechanisms by defining the degree and selectivity of back leak for ions and solutes, making an important tissue-specific contribution to overall transport (162, 209-212). TJ or zonula occludens, are the major physical structure defining the specific properties of the paracellular barrier (162, 203, 209, 211, 213-216).

Historically, the existence of transcytosis was first postulated in the 1950s by Palade in his studies of capillary permeability (217). Transcytosis is a term and concept coined by Simionescu to define the vesicular transport of macromolecules from one side of a cell to the other, a strategy used by multicellular organisms to selectively move material between two environments without altering the unique compositions of those environments (218). It is a basic process shared by most epithelial cells including endothelial cells. In intestinal cells, transcytosis is a branch of the endocytic pathway, with cargo being internalized via receptor-mediated (i.e., clathrin-coated) mechanisms and progressively sorted away from internalized material destined for other cellular destinations (134). However, transendothelial transport in blood capillaries does not

conform to this scenario, since different carriers and a more direct route are used to cross the cell. Such differences illustrate that multiple transcytotic mechanisms have evolved that depend on the particular cellular context (169). In polarized epithelial cells, net movement of material can be in either direction, apical to basolateral or the reverse, depending on the cargo and particular cellular context of the process. Moreover, reports of cultured osteoclasts (219, 220) and neurons (221) carrying vesicular cargo between two environments indicate that the strategy of vesicular transcytosis is used in other cells. It has been observed that various cargoes use different receptors that are localized to different entry sites in the plasma membrane (PM). The nature of the transcytotic cargo also varies and is not limited to macromolecules. Several vitamins and ions utilize endocytic mechanisms and vesicular carriers as part of their transcellular sojourn. Dietary vitamin B-12 (cobalamin) uses vesicle-mediated steps, in part, to cross intestinal cells. Many minerals are assumed to be absorbed paracellularly (222). Dietary iron is transported across the intestinal epithelium via multiple membrane transporters; once in the circulation, its delivery to the brain and fetus requires transcytosis. Additionally, Copper and Zinc, as well as other heavy metals, appear to be transported into intestinal absorptive cells via membrane transporters at the apical plasma membrane (169). Moreover, studies show that the kidney proximal tubule cells provide an important function in vitamin homeostasis by avidly scavenging several vitamins from the urine using a modified type of transcytosis (169).

Trafficking Pathways in Proximal Tubule Cells

The renal proximal tubule of the renal cortex is the major site for the uptake of apoA-I, HDL and most other proteins filtered in the glomeruli (22, 101). Christensen

et al. suggested that a large fraction of proteins reabsorbed by endocytosis into renal proximal tubule cells was transported intact through the proximal tubule cells by transcytosis (125). Numerous studies show that an extensive transcytosis from the apical membrane to the basolateral surface was observed in cultured rabbit proximal tubule cells (148) and in MDCK cells (223-228), constituting about 50% of the protein uptake without involvement of lysosomes and the golgi apparatus (124). Furthermore, Marino and co-workers showed that the passage of RBP (Plasma retinol-binding protein) through polarized immortalized rat renal proximal tubule (IRPT) cells occurs by transcytosis after megalin-mediated endocytosis (229). In addition, studies have shown receptor mediated endocytosis of ligands, such as cynocobalamin (193) and immunoglobulin G (230), in polarized proximal tubular epithelial cells. Nielsen and co-workers detected a very large transport of the iodinated linear peptide harmone PYY through the renal tubular epithelium (246). Ramalingam *et al.* observed immunoglobulin G transcytosis and recycling by neonatal Fc receptor (FcRn) in polarized MDCK cells (231). In another study, cultured polarized opossum kidney cells were used to study apical and basolateral uptake and metabolism of insulin (129). In the proximal tubule, filtered insulin binds to the apical membrane and is internalized and degraded. They observed that most of the internalized insulin traversed the degradative pathway but some insulin followed a retroendocytic or minor transcytotic pathway (129). A study using polarized canine proximal tubule cells by Goligorsky and Hruska provides very strong evidence for the existence of vectorial (luminal-to-basolateral) transcytosis in cultured PTC (148). Maack and Kinter originally suggested a transcellular transport of lysozyme in flounder kidney tubules initially with lysozyme followed by release after incubation (232). Later, similar

transport was found in mouse kidneys by cell fractionation techniques (233). Ottosen and Maunsbach, in similar experiments, observed that peroxidase located initially in proximal tubules was released during incubation (234). However, Ottosen demonstrated in a similar experimental system that passage of colloidal tracers from the tubule lumen into the intracellular spaces only occurs under conditions of increased intraluminal pressure by paracellular transport through TJ (235). In isolated and microperfused developing rabbit proximal tubules, paracellular transport of microperoxidase also took place through the junctional complexes when perfused at high intraluminal pressure (236). In conclusion, two pathways may exist for transfer of large molecules from the lumen to the contraluminal compartment (124, 198, 212).

Senault *et al.* explored the mechanisms of HDL association with renal cell membranes (103). While HDL could be first filtered and then endocytosed at the brush border pole of renal proximal tubular cells, a specific binding site is more likely to be located on the basolateral membrane through which HDL could be taken up directly from the circulation. Using a homologous system of porcine HDL and porcine kidney basolateral membranes, they have evidenced an interaction of HDL with the basolateral membranes of the tubular zone (103). They also identified that a 95 kDa membrane protein is responsible for the specific binding of HDL through an interaction with apoA-I. They also observed that a non-specific interaction also occurs between the lipid moiety of the particle and the lipids of the membrane (103). In addition, there is evidence, in renal epithelial cells, of symmetrical handling of lipoproteins. Streather *et al.* used polarized pig renal proximal tubule epithelial cells (LLC-PK1) to study the uptake of fluorescently labeled HDL by the apical and basolateral surfaces, while receptor-independent effects

were assessed by use of tetranitromethane treated HDL (96). They observed that HDL can enter renal epithelial cells from the apical surface. Their results with nitrosylated HDL and HMG-CoA blockade suggest that these effects may be mediated via receptors and this enzyme system (96). Furthermore, immunofluorescence studies of frozen sections of rat kidney demonstrated the presence of apoA-I on the brush-border and in apical granules of proximal tubule epithelial cells (16, 85). Peterson *et al.* studied the renal tubular reabsorption and hydrolysis of plasma HDL_3 using rabbit proximal straight nephron segments, which were microperfused *in vitro* with iodinated HDL_3 (104). Collectively, the data showed that plasma HDL_3 can be reabsorbed in the proximal nephron by a mechanism involving endocytosis at the luminal membrane, followed by proteolysis at lysosomes (104). In contrast, Remaley and co-workers found that the polarized secretion of apolipoproteins (apoA-I and apoA-II) in transfected polarized MDCK cells was not due to transcytosis, diffusion, or differential recovery, but by a cell-dependent default pathway that leads to both apical and basolateral secretion in MDCK cells, with a bias toward apical secretion (237). Despite considerable research on HDL, relatively little is known about the intracellular metabolism of this important lipoprotein class in human proximal tubule epithelial cells.

Rationale and Aims

It is well established that accelerated apoA-I catabolism is the major metabolic predictor of HDL levels (12, 238). It has been shown that plasma apoA-I levels are determined by the rate of apoA-I catabolism rather than its production (12, 15, 238). The kidney plays an important role in HDL clearance and may be quantitatively the most important site for the catabolism of HDL particles (22, 96-98). The proximal tubule is the

major site for reabsorption of filtered lipid-free apoA-I and small HDL particles to prevent its loss in the urine (96, 99, 101-104). The factors that govern their reabsorption and subsequent metabolic fate in the kidney still remain unclear.

Previous work in our laboratory has shown that HDL composition affects the reabsorption and degradation of HDL in the kidney by affecting its metabolism in proximal tubule epithelial cells. Well characterized polarized *in vitro* model systems would simulate the *in vivo* condition, which can be used to explore the molecular mechanisms of polarized membrane trafficking pathways (155, 169). Despite extensive research on membrane trafficking pathways in proximal tubule epithelial cells (130, 148, 223-228, 239), relatively little is known about the intracellular metabolism of HDL in human proximal tubule epithelial cells.

This study will attempt to characterize the factors that control the reabsorptive salvage of HDL in the kidney. The aim of the present work is to determine the role that the proximal tubule cells play in the intracellular metabolism of HDL and to determine how the biophysical properties of this lipoprotein affect the reabsorption/catabolism of apoA-I and HDL in these cells. This work will explore how HDL composition and structure regulates the intracellular metabolism of HDL in polarized human proximal tubule cells.

Hypothesis

1. Proximal tubule cells of the kidney play important roles in the reabsorptive salvage of HDL from the glomerular filtrate.
2. The proximal tubule cell plays an important role in the degradation of HDL in the kidney.
3. The structure and composition of HDL affects the reabsorption and degradation of HDL in the kidney.

Chapter 2: Experimental Procedures

Materials

[^{3}H]-FC, [^{3}H]-dipalmitoylphosphatidylcholine (DPPC) were purchased from Amersham Biosciences (Oakville, Ontario, Canada). I^{125} was purchased from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec). Egg phosphatidylcholine (PC), liver phosphatidylinositol (PI) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) were purchased from Avanti Polar Lipids (Birmingham, AL). CE was obtained from Sigma Chemical Co. (ST. Louis, MO), while DG was obtained from Nuchek Prep, Inc. (Elysian, MN). Bovine fibronectin was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Iodobeads were purchased from Pierce (Rockford, IL). Falcon Transwell tissue-culture inserts (3 μm, high pore-density (HD), polyethylene terephthalate (PET) membranes) were obtained from VWR-Canlab (Ville Mont-Royal, Quebec). Dulbecco's Modified Eagle's Medium (DMEM), F12, fetal bovine serum (FBS), bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin, L-glutamine and penicillin-streptomycin (PEN-STREP) were purchased from Gibco. All other reagents were analytical grade. Novex precast gels were purchased from Invitrogen life technologies. Monoclonal antibodies 5F6 and 4H1 against human apoA-I were a generous gift from Dr. Yves Marcel (The Heart Institute Lipoprotein Research Group, University of Ottawa, Ontario). The HKC-8 kidney proximal tubule cell line was obtained from the laboratory of Dr. Lorraine Racusen (John Hopkins School of Medicine, Baltimore, MD).

Methods

I. Preparation of Ligands

Isolation of Human HDL and HDL fractions

Human HDL (ρ =1.063-1.210 g/ml) and various HDL fractions (HDL_2 - ρ = 1.063-1.125 g/ml; HDL_3 - ρ = 1.125-1.21 g/ml; VHDL - ρ = 1.21-1.25 g/ml) were isolated from fresh plasma by sequential density gradient ultracentrifugation according to the procedure of Havel *et al.* (240).

Purification of ApoA-I

Human HDL was delipidated using a chloroform:methanol extraction as previously described (241). Purified apoA-I was isolated by size-exclusion chromatography on a Sephacryl S-200 HR column (242). ApoA-I was stored in lyophilized form at –80 °C. Prior to use, it was resolubilized in 6 M guanidine hydrochloride and 10 mM Tris, pH 7.2, and dialyzed extensively against PBS. Protein concentrations were determined using the Lowry method as modified by Markwell (243).

Iodination of ApoA-I, HDL and Various HDL Fractions

Purified apoA-I, human HDL and various HDL fractions were iodinated with ^{125}I using the IODO-BEAD Iodination reagent (Pierce; Rockford, IL) and manufacturer-recommended protocols. The labelled HDL was dialyzed extensively against PBS to remove any non-protein associated ^{125}I.

Preparation of Reconstituted Spherical HDL Particles

Reconstituted HDL particles (rHDL) were prepared by co-sonication of fixed ratios of pure or extracted HDL lipids and apoA-I or total HDL apolipoproteins (apoHDL). Briefly, specific amounts of lipids in chloroform (see Table 3 for molar ratios) were dried under

nitrogen in a 12 x 75 mm glass tube, and 800 µl of PBS was added. The lipid-buffer mixture was successively sonicated with a Branson Sonifier 450 (Branson Ultrasonics, Danbury, CT) under nitrogen for 1 min at constant output, incubated at 37 °C for 30 min and sonicated again for 5 min at 95% duty cycle under nitrogen. ^{125}I-apoA-I (0.5 mg) and unlabeled apoA-I (diluted to 1.4 mg/ml in PBS) were added to the lipid mixture and co-sonicated for 4 x 1 min at 90% duty cycle under nitrogen, with 1 min cooling periods between sonications. The particles were then filter sterilized through a 0.22 µm filter. The size and homogeneity of the particles were estimated by non-denaturing gradient gel electrophoresis (Pharmacia, Phast System). Particle charge was determined from electrophoretic mobility in 0.5 % agarose gels containing BSA (Beckman, Paragon Lipo kit).

FC (Wako Chemicals USA, Richmond, VA), PL (Roche Diagnostics, Laval, PQ) and protein (Pierce, Rockford, IL) were determined enzymatically using commercial test kits. In addition, following a chloroform:methanol extraction (241), both lipids and proteins from HDL particles were recovered and used to make reconstituted particles.

Table 3: Reconstituted HDL Composition

Particle[a]	Particle Constituents	PC:CE:apoA-I (mol:mol:mol) [b]
rHDL-CE	POPC:CE:apoA-I	60:30:1
rHDL-lipids	HDL lipid*:apoA-I	60:0:1
rHDL-apo	POPC:apoHDL**	60:0:1

*HDL lipid was prepared by solvent extraction of human HDL
** ApoHDL was prepared by re-solubilization of the precipitated HDL proteins after solvent extraction of HDL
[a] Spherical rHDL particles were prepared by sonication as described in the text.
[b] Composition determined as described of phosphatidylcholine (PC), cholesteryl ester (CE) and apoA-I.

Preparation of [^{3}H]-cholesterol and [^{3}H]-DPPC HDL

One hundred μl (100 μCi) of [^{3}H]-cholesterol was spotted onto a disk of filter paper, dried under nitrogen and incubated for 24 h at 4°C with HDL (300 μg/ml) mixture. Fifty μl (50 μCi) of [^{3}H]-DPPC was dried under nitrogen in a 12 x 75 mm glass tube, and 800 μl of PBS was added. The lipid-buffer mixture was successively sonicated with a Branson Sonifier 450 (Branson Ultrasonics, Danbury, CT) under nitrogen for 1 min at low output and constant duty cycle while cooling in a water bath, incubated at 37°C water bath for 15 minutes. Sonicated again for 3 min at high output and 95% duty cycle under nitrogen and was incubated for 24 h at 4°C with HDL (300μg/ml).

Preparation of Phospholipid Vesicles and Phospholipid Enriched HDL

POPC and PI vesicles in PBS (1 mg/ml) were prepared by sonication as previously described (67). To prepare PL enriched HDL, the lipoprotein was incubated for 24 h at 4°C with PC or PI vesicles. To label HDL, the appropriate amount of [^{3}H]-FC was spotted onto a disk of filter paper, dried under nitrogen and included in the lipid-HDL incubation.

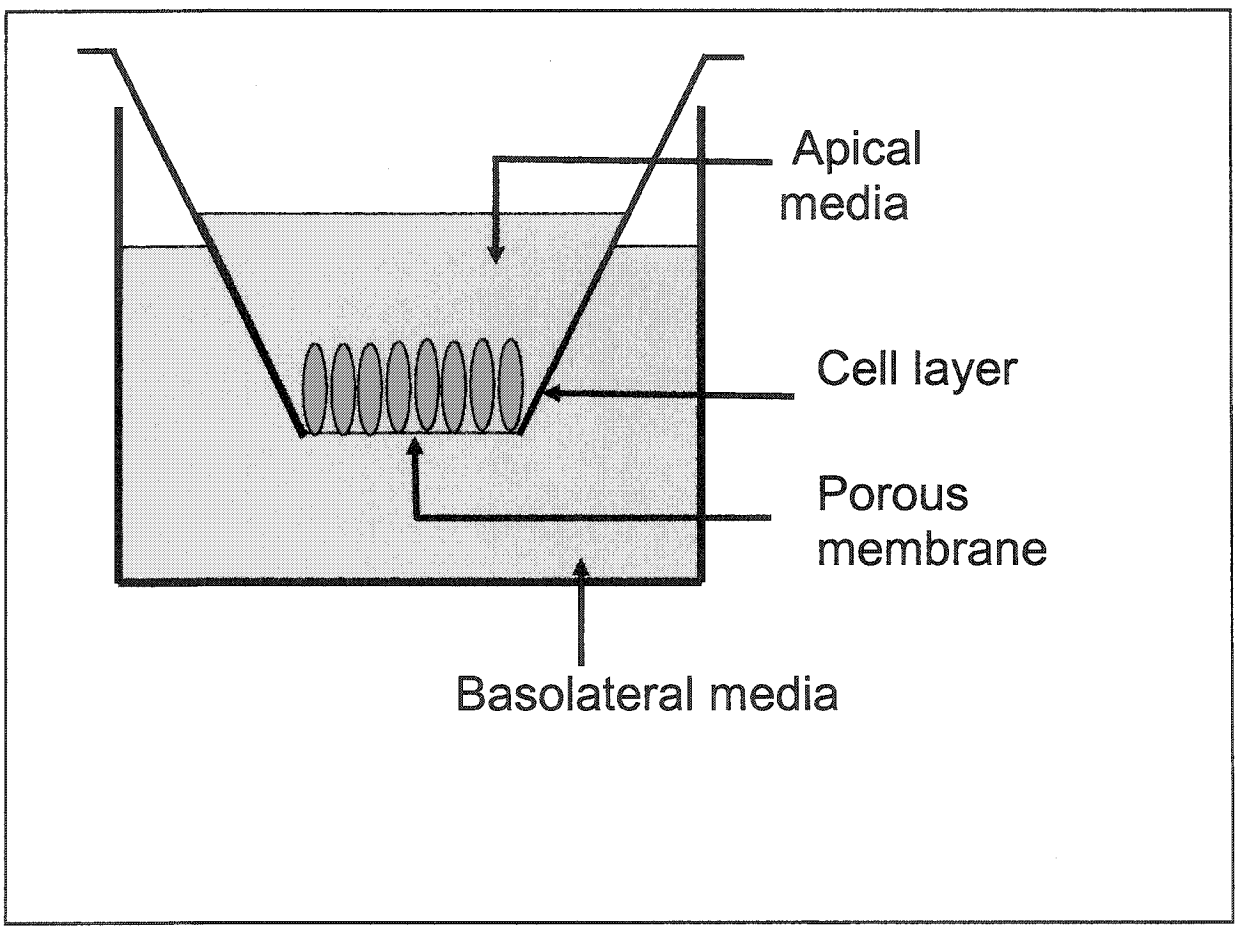
II. *In vitro* Experiments with Polarized Cells

Cell Culture

HKC-8 cells were seeded into 12-well Falcon plates and grown to confluence in a CO_2 incubator at 37°C (Fig. 5). The cells were cultured in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The Transwell tissue-culture inserts were pre-coated with 30 μg/ml fibronectin in DMEM to a final coating concentration of 3 μg/cm^2 according to manufacturer-recommended protocols. The cells

Figure 5: Polarized Cells in Transwell Tissue-Culture Inserts

Epithelial cells become morphologically and functionally polarized when grown as a monolayer on a Transwell tissue-culture insert. A porous membrane in the insert separates the upper apical compartment from the basolateral surface. This model can be used to study the uptake and transport of lipoproteins within the cultured cells.


Apical
media
Cell layer
Porous
membrane
Basolateral media

were then plated onto fibronectin-coated tissue-culture inserts and allowed to grow for 4 days in DMEM/F12 supplemented with 2% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Fig. 5).

Ligand Processing and Degradation Assays

Growth medium was removed and cells were washed twice with serum-free DMEM/F12. 0.5 ml of ^{125}I-labeled ligand in DMEM/F12 media (5 mg/ml BSA, 1 mM $CaCl_2$) was added to the top compartment (apical) and 1.5 ml of ligand-free media was added to the bottom compartment (basolateral) at a final concentration of 300 μg protein/ml per well. The cells were then incubated at 37°C for various time points. At the end of each time-point, medium was collected at 4°C from both chambers for counting. The cells were washed twice with PBS containing 1 mM $CaCl_2$. The cells were then lysed in 0.2 M NaOH, overnight at room temperature, with gentle rocking. Following the incubation, lysates were removed for counting and cell protein determination. For degradation analysis, an aliquot of media post-incubation was added to an equal volume of 25% TCA for precipitation. The radioactivity of the soluble products was measured and expressed as the percentage of total radioactivity.

In order to monitor the integrity of the confluent monolayers throughout the experiment, [^{14}C]-inulin (0.1 μCi) was added to apical compartments of a separate set of wells for each time point. The presence of [^{14}C]-inulin in the apical and basolateral compartments was then monitored by scintillation counting. Fibronectin-coated Transwells without cells were used as controls at each timepoint.

Ligand Internalization Assay

Growth medium was removed and cells were washed twice with serum-free DMEM/F12. 0.5 ml of ^{125}I-labeled ligand in DMEM/F12 media (5 mg/ml BSA, 1 mM $CaCl_2$) was added to the top compartment (apical) and 1.5 ml of ligand-free media was added to the bottom compartment (basolateral) at a final concentration of 300 μg/ml per well. The cells were then incubated at 37°C for 6 h. At the end of the time-point, medium was collected at 4°C from both chambers for counting. The cells were washed twice with PBS containing 1 mM $CaCl_2$. The cells were treated with trypsin at 4°C for an hour. After an hour the cell pellet was separated from the supernatant. The cell pellets were then washed with PBS and lysed in 0.2 M NaOH, overnight at room temperature, with gentle rocking. Following the incubation, lysates were removed for counting and cell protein determination. For degradation analysis, an aliquot of media post incubation was added to an equal volume of 25% TCA for precipitation.

Pulse-Loading Assay

Growth medium was removed and cells were washed twice with serum-free DMEM/F12. 0.5 ml of ^{125}I-labeled ligand in DMEM/F12 media (5 mg/ml BSA, 1 mM $CaCl_2$) was added to the top compartment (apical) and 1.5 ml of ligand-free media was added to the bottom compartment (basolateral) at a final concentration of 300 μg/ml per well. The cells were then incubated at 37°C for an hour (pulse). After one hour, cells from a plate designated as t =0 were harvested. To the remainder, medium was collected and cells were washed twice with serum-free DMEM/F12. 0.5 ml of ligand free media was added to the top compartment (apical) and 1.5 ml of ligand-free media was added to the bottom compartment (basolateral). The cells were then incubated at 37°C for various

time points. At the end of each time-point, medium was collected at 4°C from both chambers for counting and protein determination. The cells were washed twice with PBS containing 1 mM $CaCl_2$. The cells were then lysed in 0.2 M NaOH, overnight at room temperature, with gentle rocking. Following the incubation, lysates were removed for counting and cell protein determination. For degradation analysis, an aliquot of media post incubation was added to an equal volume of 25% TCA for precipitation. The radioactivity of the soluble products was measured and expressed as the percentage of total radioactivity. The media was analyzed using SDS-PAGE and native PAGE. Plasma apoA-I was determined by quantitative Western blotting.

In order to monitor the integrity of the confluent monolayers throughout the experiment, [^{14}C]-inulin (0.1 μCi) was added to apical compartments of a separate set of wells for each time point. The presence of [^{14}C]-inulin in the apical and basolateral compartments was then monitored by scintillation counting. Fibronectin-coated Transwells without cells were used as controls at each timepoint.

Western Blotting

Western blots for apoA-I were performed on the apical and basolateral media after it was subjected to 12% SDS-PAGE under reducing conditions or 4-20% polyacrylamide gradient gel electrophoresis (PAGGE) under native conditions. Samples separated by 12% SDS and 4-20% PAGGE were transferred at 125 V for 2 h on to nitrocellulose membranes. The membranes were blocked overnight in blocking solution (5% skim milk, 0.2% Tween, 0.02% azide and PBS) at 4°C. The nitrocellulose membranes were probed with anti-apoA-I antibodies 4H1 and 5F6, following incubation with sheep anti-mouse IgG (Amersham Biosciences, Inc.) coupled to HRP. Proteins were

visualized by chemiluminescence with the Pierce West Pico SuperSignal substrate as described.

Ligand Uptake Assay

Growth medium was removed and cells were washed twice with serum-free DMEM/F12. Ligand in 0.5 ml of DMEM/F12 media (supplemented with 5 mg/ml BSA, 1mM $CaCl_2$) was added to the top compartment (apical) and 1.5 ml of ligand-free media was added to the bottom compartment (basolateral) at a final concentration of 300 μg/ml per well. The cells were then incubated at 37°C for 2 hours. At the end of the time-point, medium was collected at 4°C from both chambers for protein determination. The cells were washed twice with PBS containing 1 mM $CaCl_2$. Cells from a plate were washed with a mild acid wash (207). SDS sample buffer was added to all the cells and incubated at 37°C for 30 minutes. Following the incubation, lysates were removed for ligand protein determination. Western blots for apoA-I were performed on an aliquot of the cell lysate after it was subjected to 12% SDS-PAGE under reducing conditions. Molecular weight of apoA-I is 28 kDa. The basolateral media was analyzed using SDS-PAGE and Native PAGE. Plasma apoA-I was determined by quantitative Western blotting.

Cholesterol and PL uptake assays were performed similar to the pulse-loading assay but with the following necessary modifications. Growth medium was removed and cells were washed twice with serum-free DMEM/F12. [^{3}H]-FC or [^{3}H]-DPPC-labeled ligand in 0.5 ml of DMEM/F12 media (supplemented with 5 mg/ml BSA, 1 mM $CaCl_2$) was added to the top compartment (apical) and 1.5 ml of ligand-free media was added to

the bottom compartment (basolateral) at a final concentration of 300 μg/ml per well. The rest of the experiment was done similar to the pulse-loading experiment described above.

PL enriched HDL processing assay was also evaluated similar to the pulse-loading assay but with the following modifications. Growth medium was removed and cells were washed twice with serum-free DMEM/F12. [^{3}H]-FC-labeled HDL, PI- or PC-enriched and [^{3}H]-FC labeled (prepared as described) in 0.5 ml of DMEM/F12 media (5 mg/ml BSA, 1 mM $CaCl_2$) was added to the top compartment (apical) and 1.5 ml of ligand-free media was added to the bottom compartment (basolateral) at a final concentration of 300 μg/ml per well. The rest of the experiment was done similar to the pulse-loading experiment described above.

Western blots for apoA-I were performed on an aliquot of the apical and basolateral media at the 2 hour timepoint after it was subjected to 12% SDS-PAGE under reducing conditions.

Statistical Analysis

Using GraphPad InStat software version 3.00, a one-way ANOVA statistical analysis was performed on the data sets to obtain significance of difference between sample means. $p<0.001$ was regarded as statistically significant.

Chapter 3: RESULTS

In vitro Experiments with Polarized Cells

Rationale

Numerous tissue uptake studies have shown that HDL can be filtered by the kidneys and that radioactively labelled HDL components can be found in the kidney cortex (16-21, 85). While the general consensus is that lipid poor apoA-I is filtered by the kidneys, some studies suggest that the kidneys are capable of both the filtration and re-absorption of apoA-I and small-sized HDL particles (85, 100, 104).

We have chosen to utilize a cell culture system, HKC-8 cells, using an immortalized human renal tubule epithelial cell line. The cell line has been shown to maintain normal expression of tubule markers such as alkaline phosphatase, χ-glutamyl transpeptidase or glutathione-S-transferase, maintain normal biochemical properties and display normal epithelial monolayer morphology with a well-developed brush border for prolonged periods. The HKC-8 cell line is comparable to established cell lines such as opossum kidney cell line (OK), human kidney cell line (HK-2), and rabbit kidney epithelial cell line (LLC-RK1) (190).

An essential feature of these cells is their polarity. In the case of absorptive epithelia, such as that of the intestine or the renal tubule, their luminal (apical) surface is exposed to a fluid filled space while their basal surface is attached to the basement membrane above the underlying vascular tissues. The two membranes differ morphologically, functionally and biochemically. This can be recreated *in vitro* by culturing epithelial cells on porous polycarbonate Transwell filters. Pre-coating these filters with ECM components, such as fibronectin or collagen, and establishing the proper

media conditions can dramatically alter the morphology of epithelial cells thereby enabling them to display polarity and reach a well-differentiated state (158). While much valuable information may be obtained from studying epithelial cells cultured with conventional (solid surface) *in vitro* techniques, such differences emphasize the importance of studying these cells in a polarized environment, as this would more closely resemble the *in vivo* situation. The focus of this work was to study the intracellular metabolism of HDL by polarized HKC-8 cells grown *in vitro* on Transwell tissue-culture inserts.

In all the experiments, HKC-8 cells were cultured on fibronectin-coated Transwell tissue-culture inserts as described in the Materials and Methods. Polarized HKC-8 cells were incubated with ligands (300 μg protein/ml) that were added to the apical compartment for various times at 37°C. To make sure that the cells formed a tightly sealed monolayer that is virtually impenetratable to small molecules, we routinely assessed [^{14}C]-inulin diffusion across the cell monolayer in the experiments described and showed that the HKC-8 cells cultured on fibronectin-coated permeable filters provide a relatively impermeable diffusion barrier to passive paracellular diffusion.

Ligand Processing and Degradation Assays with HDL Fractions

In order to identify a relationship between HDL size and intracellular metabolism, polarized HKC-8 cells were incubated with ^{125}I protein-labeled HDL_2, HDL_3 or VHDL particles (300 μg protein/ml) that were added to the apical compartment for various times. Following the incubation, the medium was removed from both compartments and counted to determine total radioactivity per compartment. The cells were washed in buffer on ice and lysed in 0.2 M NaOH. The cell lysates were then

evaluated for radioactivity by gamma counting. Figure 6 shows the time course of ^{125}I-labeled HDL fractions binding to polarized human proximal tubule cells. The results indicate that all of the HDL fractions associated with the cell surface similarly until 1 hour (Fig. 6). After 2 hours, VHDL and HDL_2 appear to associate more with the cells when compared to HDL_3. This result shows that the largest and the smallest HDL fractions associate more with the cells. In the same experiment, transport of the different ^{125}I-HDL fractions to the basolateral compartment was monitored (Fig. 7). The three HDL fractions appear to be secreted to the basolateral side in equal amounts. The results suggest that there is an equal amount of secretion with time.

To confirm that intact apoA-I is being secreted from the basolateral side of the cell, polarized HKC-8 cells were incubated with ^{125}I-HDL (300 μg protein/ml) and then the media were probed for apoA-I. Western blots for apoA-I were performed on an aliquot of the apical and basolateral media at each time point after it was subjected to 12% SDS-PAGE under reducing conditions (Fig. 8) (Molecular weight 28 kDa). The western blot clearly shows that intact apoA-I is secreted to the basolateral side. Also, we observed an increase in secretion of apoA-I with time. Overexposed Western blot showed no apparent degradation fragments. Maack and coworkers observed that a large fraction of reabsorbed protein was transported intact through the proximal tubule cells by transcytosis in their studies on flounder kidney tubules (232, 233).

In the same experiment, native acrylamide gels were run on the media to determine whether HDL was secreted from the basolateral side of the cell as a whole HDL or whether it was remodelled/degraded and resecreted (Fig. 9). We observed that when whole native HDL is added to the apical side of polarized HKC-8 cells, HDL_3 sized

Figure 6: Association of Different HDL Fractions with Polarized HKC-8 Cells

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were incubated with ^{125}I-VHDL, ^{125}I-HDL_2 or ^{125}I-HDL_3 particles (300 μg protein/ml) added to the apical compartment at 37°C for various times. The cell lysates were counted to determine total cell associated radioactivity. The data are presented as the mean ± SD of triplicate determinations. The data is representative of three experiments. A one-way ANOVA was performed to determine statistical significance of difference relative to HDL_3 values (*$p<0.001$).

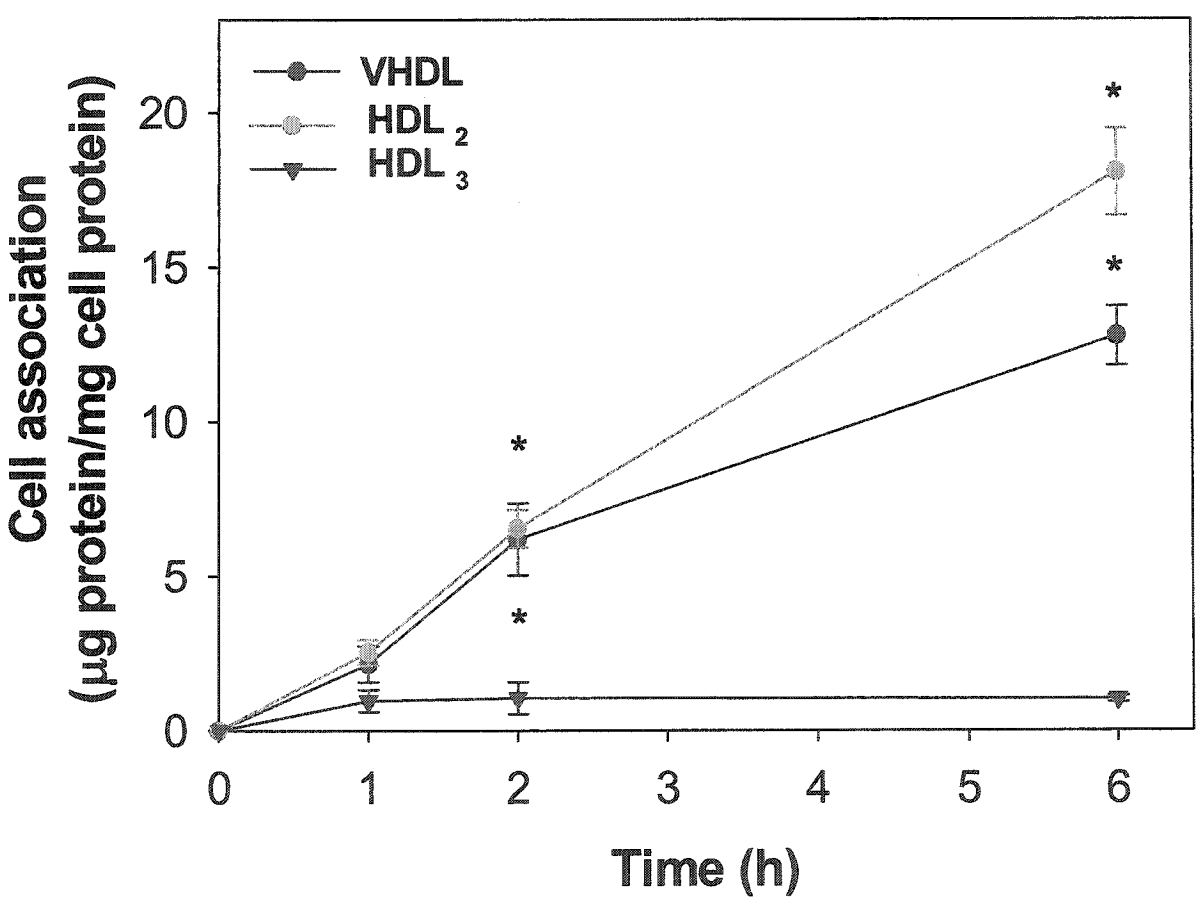

VHDL
HDL 2
HDL 3
Cell association
(µg protein/mg cell protein)
0
5
10
15
20
0
1
2
3
4
5
6
Time (h)
*
*
*
*

Figure 7: Transport of Different ^{125}I-HDL Fractions to the Basolateral Compartment

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were incubated with ^{125}I-VHDL, ^{125}I-HDL_2 or ^{125}I-HDL_3 (300 μg protein/ml) added to the apical compartment at 37°C for various times. Following the incubation, the medium was removed from both compartments and counted to determine total radioactivity per compartment. An aliquot of the media at each time point was analyzed for degradation products by precipitation in 25% TCA. Transport to the basolateral compartment was determined as the difference between the total transport and non-precipitable radioactivity in the basolateral compartment. The data are presented as the mean ± SD of triplicate determinations. The data is representative of three experiments.

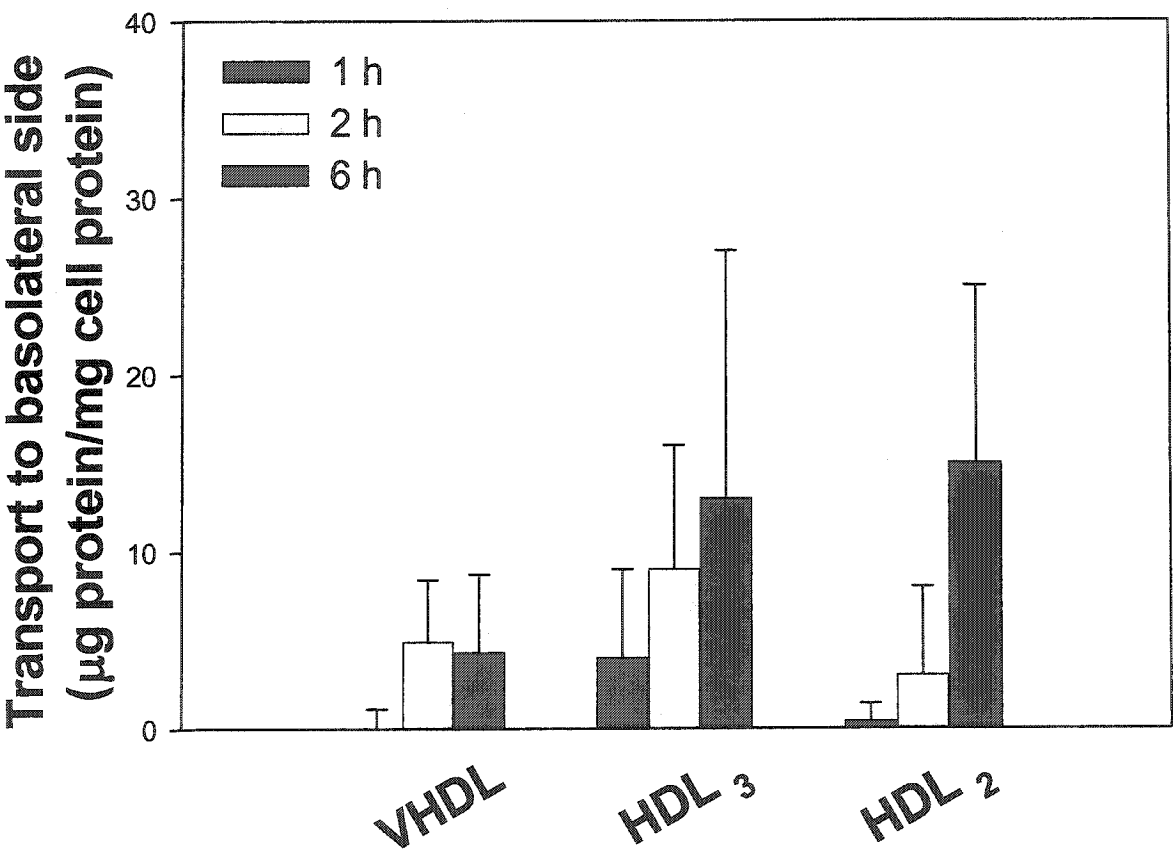

Transport to basolateral side
(µg protein/mg cell protein)
40
30
20
10
0
1 h
2 h
6 h
VHDL
HDL 3
HDL 2

Figure 8: ApoA-I is Transported Intact

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were incubated with ^{125}I-HDL (300 μg protein/ml) added to the apical compartment at 37°C for various times. Following the incubation, the medium was removed from both compartments. Western blots for apoA-I was performed on an aliquot of the basolateral media at each time point, after it was subjected to 12% SDS-PAGE under reducing conditions. The molecular weight of apoA-I is 28 KDa. The data is representative of three experiments.

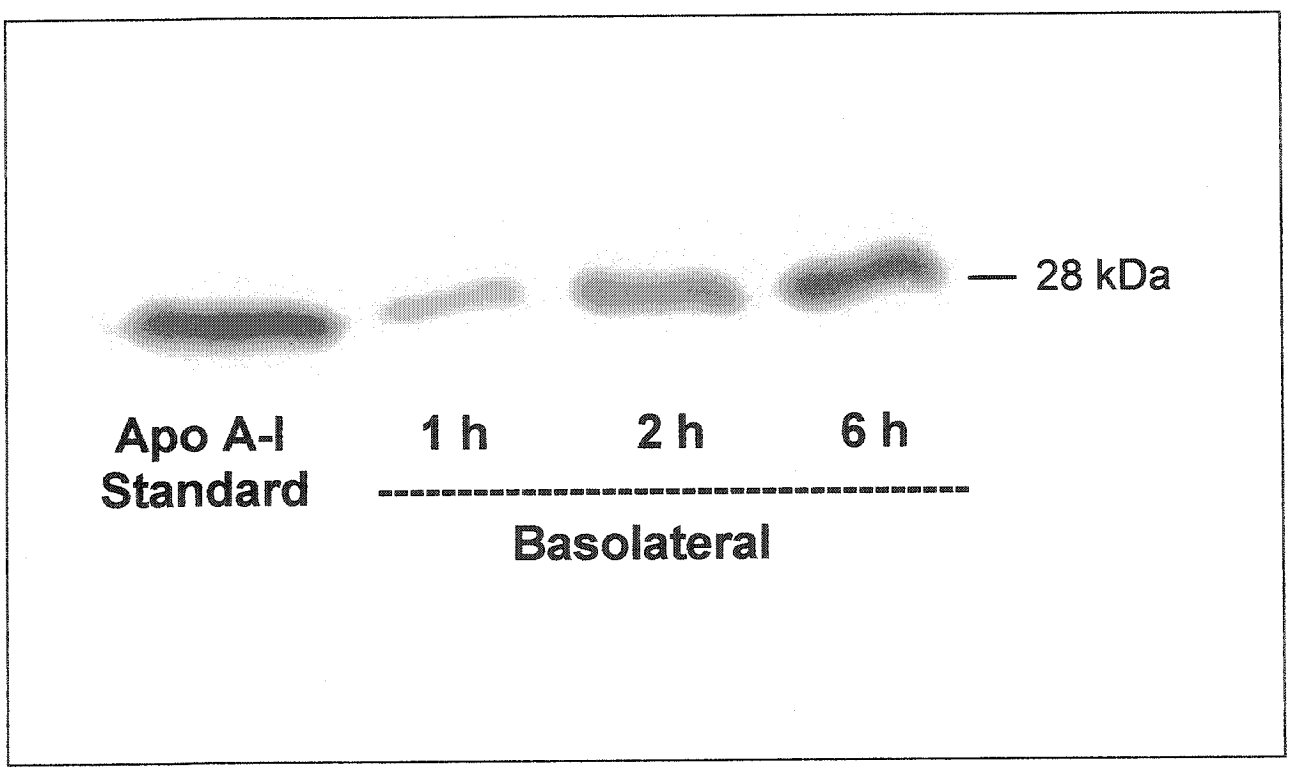
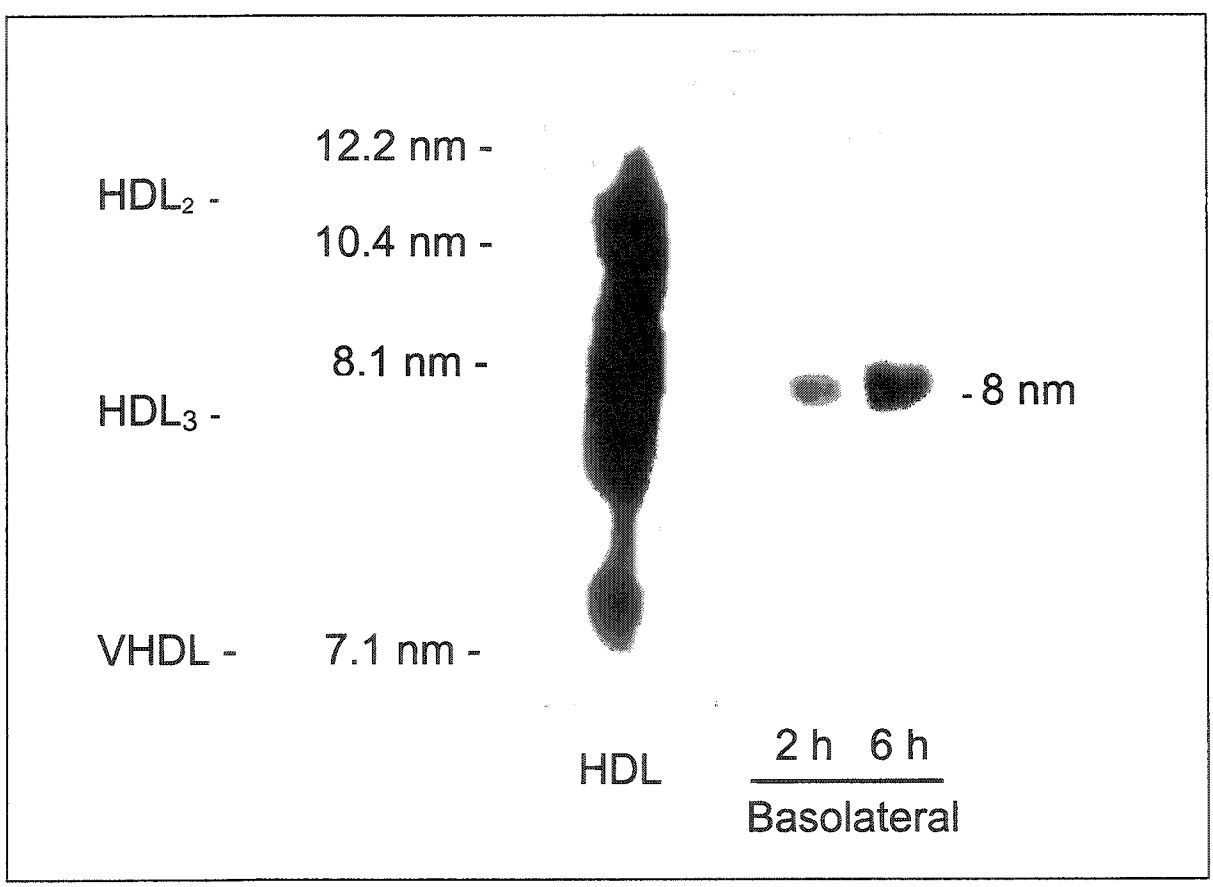

— 28 kDa
Apo A-I
Standard
1 h
2 h
6 h
Basolateral

Figure 9: Whole HDL Particles are Transported through HKC-8 Cells

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were incubated with ^{125}I-HDL (300 μg protein/ml) added to the apical compartment at 37°C for various times. Following the incubation, the medium was removed from both compartments and counted to determine total radioactivity per compartment. Western blots for apoA-I were performed on an aliquot of the basolateral media at each time point after it was subjected to 4-20% PAGGE under native conditions. The data is representative of three experiments


12.2 nm -
HDL2 -
10.4 nm -
8.1 nm -
HDL3 -
- 8 nm
VHDL -
7.1 nm -
HDL
2 h 6 h
Basolateral

particles seem to be secreted/ transported from the basolateral side (Fig. 9). An increase in secretion of HDL over time was observed, similar to the results seen in the SDS Western blot (Fig 8). Previous studies showed that intact HDL_3 is bound specifically by polarized Caco-2 cells, leading to a subsequent intracellular passage (176). Peterson *et al.* showed that plasma HDL_3 can be reabsorbed in the rabbit proximal straight nephron segments, which were microperfused *in vitro* with iodinated HDL_3 by a mechanism involving endocytosis at the luminal membrane (104). In addition, Klinger *et al.* showed that HDL_3 is internalised and subsequently processed in an endosomal pathway in Caco-2 cells (177).

Ligand Internalization Assays

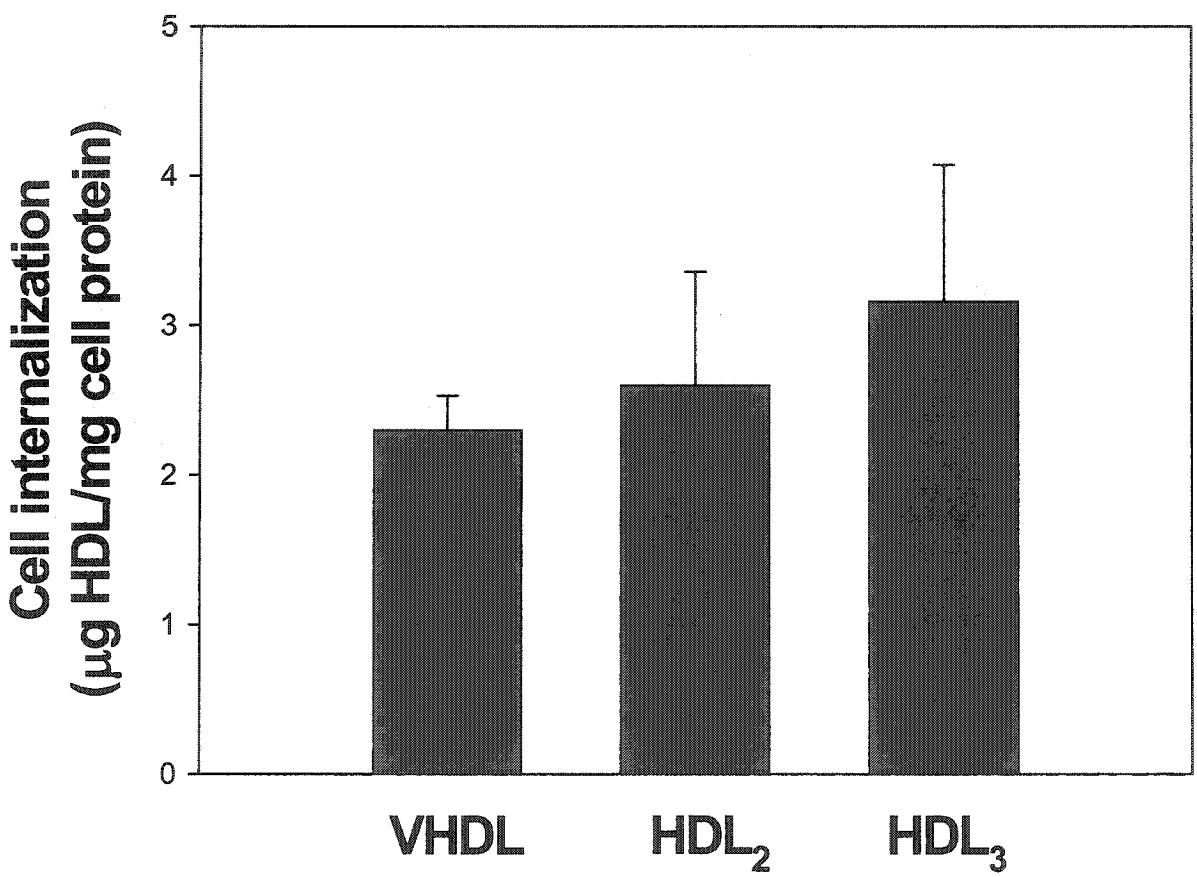
Experiments were performed to differentiate the cell-internalized and the surface-bound ligand from total cell association of different HDL fractions. Polarized HKC-8 cells were incubated with labeled HDL_2, HDL_3 and VHDL (300 μg protein/ml) added to the apical compartment for 6 hours. Following the incubation, the medium was counted to determine total radioactivity. To determine cell internalization, the cells were treated with trypsin at 4°C for 1 h and the cell pellet was separated from the supernatant. The panels show the internalization of different HDL subfractions at the 6 hour time point (Fig. 10). All three fractions appear to be internalized to the same extent.

Ligand Uptake Assay

To further compare endocytic uptake with total cell association of the three subfractions of HDL, we designed an experiment to study uptake immunochemically. Polarized HKC-8 cells were incubated with HDL_2, HDL_3 and VHDL (300 μg protein/ml) for 2 hours. Following the incubation, the medium was removed from both compartments.

Figure 10: Internalization of Various HDL Fractions from HKC-8 Cells

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were incubated with ^{125}I-HDL$_2$, ^{125}I-HDL$_3$ and ^{125}I-VHDL (300 μg protein/ml) added to the apical compartment at 37°C for 6 hours. Following the incubation, the medium was removed from both compartments. The cells were treated with trypsin at 4°C for an hour. After an hour the cell pellet was separated from the supernatant. The cell pellets were washed and lysed. The lysates were counted to determine cell internalized radioactivity. The panels show the internalization of different HDL subfractions at the 6 hour time point. The data are presented as the mean ± SD of triplicate determinations. The data is from one experiment.


Cell internalization
(μg HDL/mg cell protein)
5
4
3
2
1
0
VHDL
HDL2
HDL3

Cells were washed with an acid wash and SDS sample buffer was added to all the cells to collect the cell lysates. Western blots for apoA-I were performed on an aliquot of the cell lysate after it was subjected to 12% SDS-PAGE under reducing conditions. We observed that all the three HDL subfractions appear to be associated to the cells to a more or less similar extent in the total cell extract (Fig. 11). We further observed in the acid wash cell extract that significantly more cell uptake of HDL_3 was evident, when compared to the other HDL subfractions. We also observed that HDL_2 is not cell internalized and that VHDL is cell internalized moderately when compared to HDL_2. In the same experiment, non-denaturing acrylamide gels were run to determine whether whole HDL particles were secreted from the basolateral side of HKC-8 cells. We observed that all three subfractions of HDL were transported intact to the basolateral side (Fig 12). To compare the amounts of HDL fractions transported, Western blots for apoA-I were also performed on an aliquot of the basolateral media after it was subjected to 12% SDS-PAGE under reducing conditions. We observed that all the three subfractions of HDL were transported to the same extent (Fig 12).

Ligand Processing Assay with Labeled apoA-I, HDL and rHDL

To determine the effects of HDL composition on its intracellular metabolism, polarized HKC-8 cells were incubated with labeled apoA-I, HDL or rHDL particles (300 μg protein/ml) for various times. A chloroform-methanol organic extraction was performed to separate native HDL into its lipid and apolipoprotein constituents. The obtained HDL lipids and HDL apolipoproteins were then used to reconstitute rHDL particles with apoA-I and POPC, respectively, by co-sonication. To elucidate the effect of unique HDL lipids on rHDL particle association with HKC-8 cells, particles were

Figure 11: Intact ApoA-I is Cell-Associated and Internalized

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were incubated with HDL_2, HDL_3 and VHDL (300 μg protein/ml) added to the apical compartment at 37°C for 2 hours. Following the incubation, the medium was removed from both compartments. Cells from a plate were washed with an acid wash. SDS sample buffer was added to all the cells to collect the cell lysates. Western blots for apoA-I was performed on an aliquot of the cell lysate after it was subjected to 12% SDS-PAGE under reducing conditions. The histograms shown represent densitometric quantification of the apoA-I bands (28 kDa) shown above. The data is from one experiment.

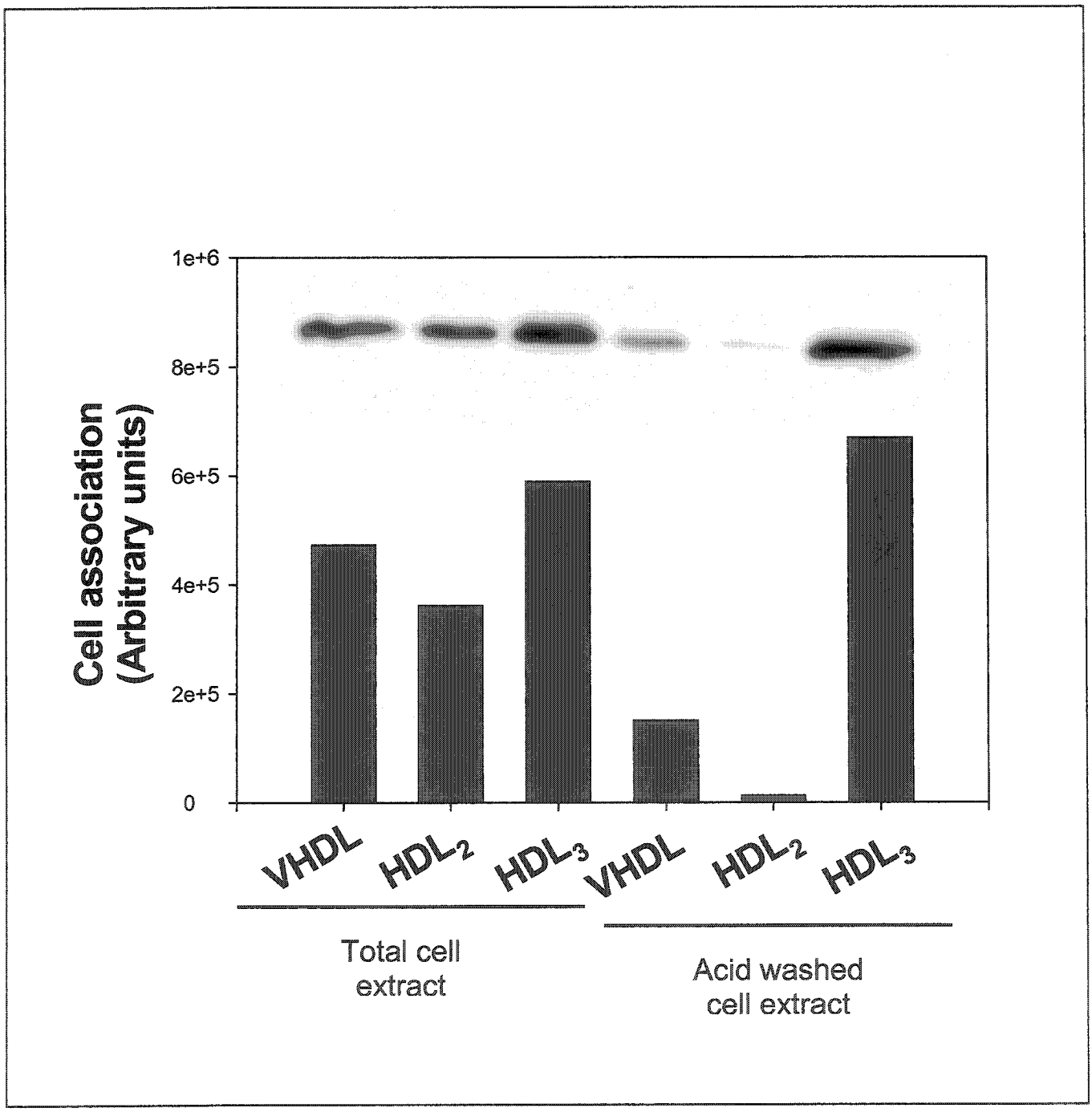
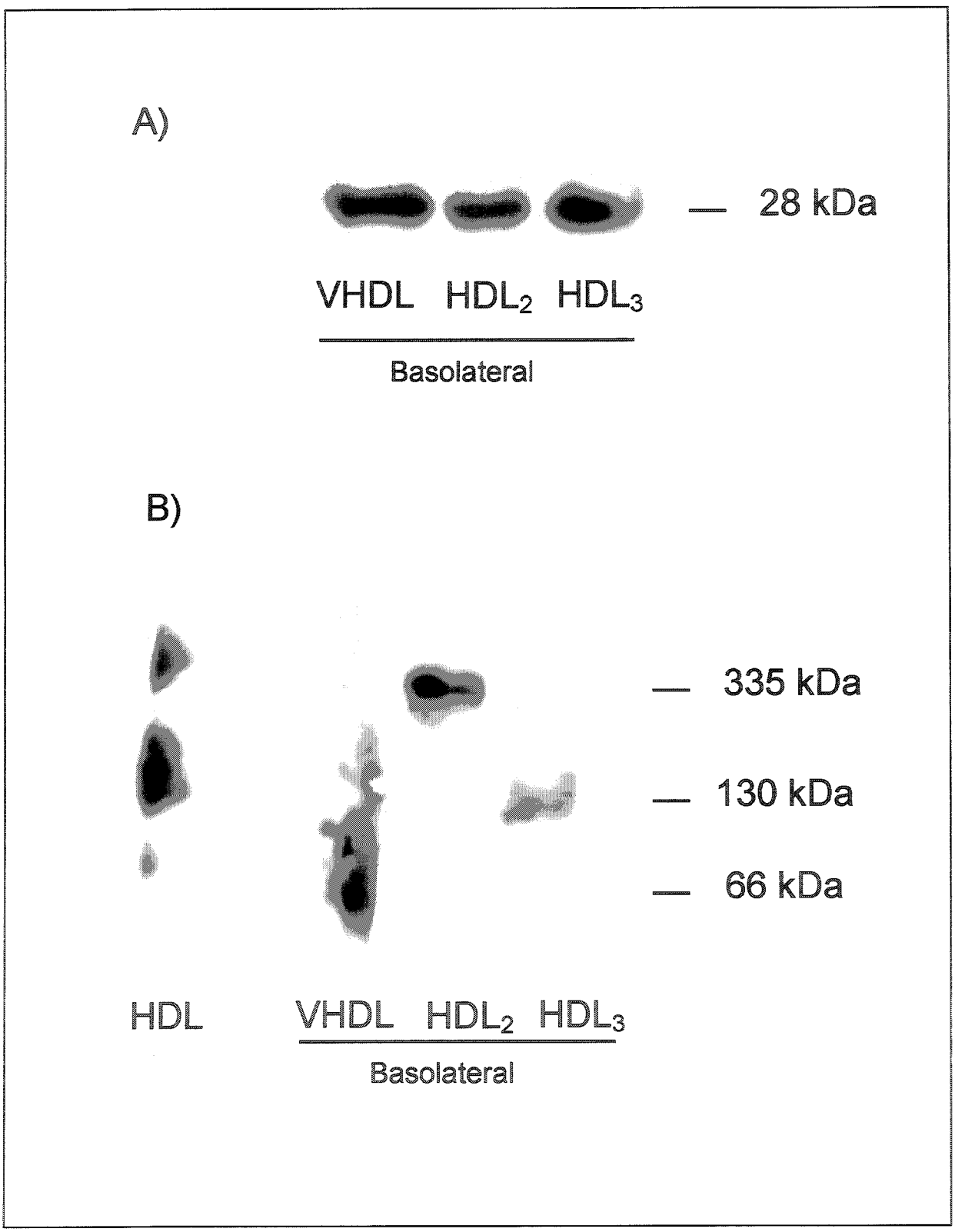

Cell association
(Arbitrary units)
1e+6
8e+5
6e+5
4e+5
2e+5
0
VHDL
HDL2
HDL3
VHDL
HDL2
HDL3
Total cell
extract
Acid washed
cell extract

Figure 12: Whole HDL Fractions are Transported through HKC-8 Cells

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were incubated with HDL_2, HDL_3 and VHDL (300 μg protein/ml) added to the apical compartment at 37°C for 2 hours. Following the incubation, the medium was removed from both compartments. Cells from a plate were washed with an acid wash SDS sample buffer was added to all the cells to collect the cell lysates. Panel A: Western blots for apoA-I was also performed on an aliquot of the basolateral media after it was subjected to 12% SDS-PAGE under reducing conditions. Panel B: Western blots for apoA-I was performed on an aliquot of the basolateral media after it was subjected to 4-20% PAGGE under non-denaturing conditions. The data is from one experiment.

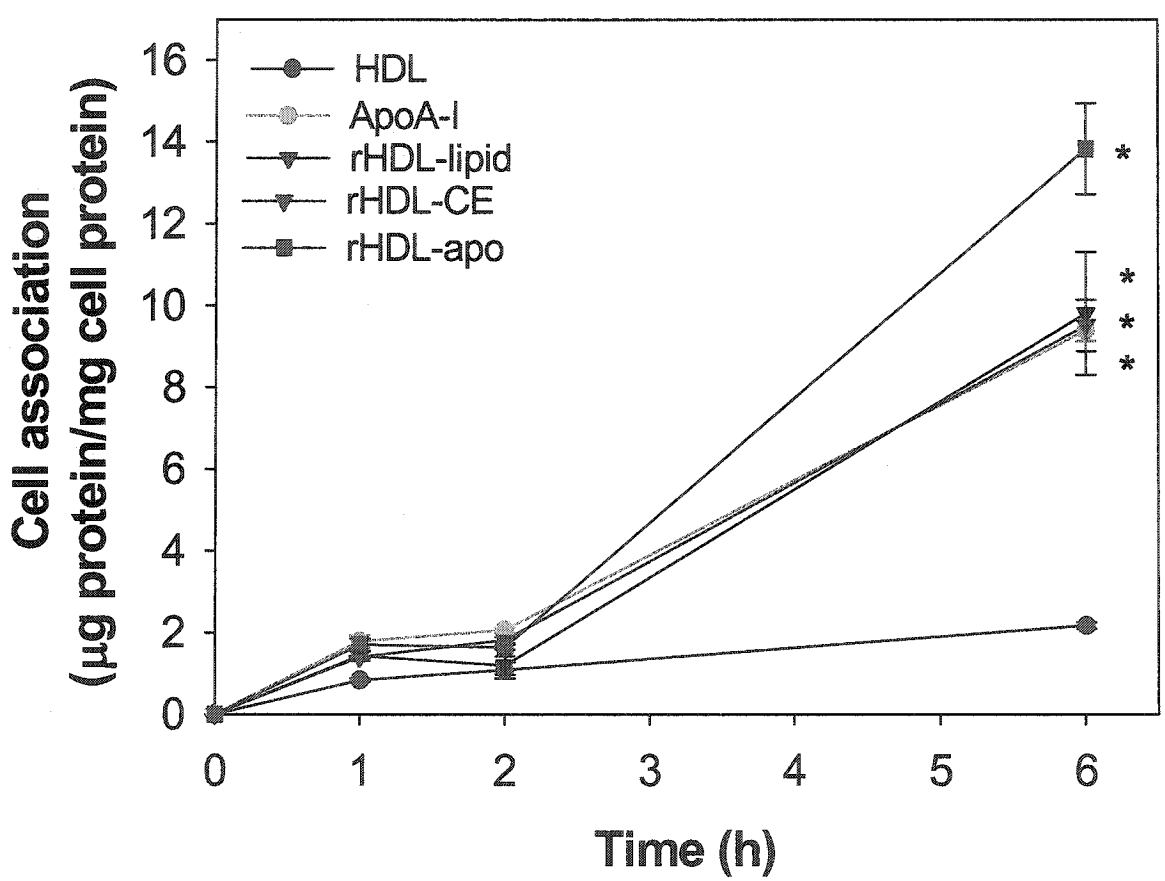

A)
28 kDa
VHDL HDL2 HDL3
Basolateral
B)
335 kDa
130 kDa
66 kDa
HDL
VHDL HDL2 HDL3
Basolateral

reconstituted with cholesteryl linoleate (CE). Previous experiments in our lab have shown that ^{125}I-rHDL reconstituted with CE had a 2-fold increase in specific cell association when compared to that of ^{125}I-rHDL containing no CE (244). Previous studies have also demonstrated a structural effect of CE on HDL particles and shown an increase in the negative surface charge and stability of the lipoprotein complexes and an increase in the content of α-helical structures in apoA-I (14). Figure 13 represents a comparison of the total cell association of various lipoprotein particles with polarized HKC-8 cells over time. The figure shows an increase in cell association with ^{125}I-apoA-I, ^{125}I-HDL and ^{125}I-rHDL particles until 1 hour after which it plateaus until 2 hours. The figure further shows that all the ligands associate with the cells to a similar extent up to 2 hours. Furthermore, we observed that after 2 hours there is an increase in cell association with all the ligands except with native HDL. In addition, rHDL-apo seems to associate with the cells to the greatest extent and native HDL seems to cell associate the least when compared to the various reconstituted particles and apoA-I at 6 hours (Fig. 13). In contrast, several laboratories have demonstrated very high ^{125}I-HDL binding to the membranes of the renal cortex of various species, including those of human, porcine and rat origin (97, 98, 103). Pure ^{125}I-apoA-I has also been shown to compete less effectively for HDL binding sites than ^{125}I-HDL$_3$ in human enterocytes (245).

In the same experiment, the transport of labeled apoA-I, HDL and rHDL particles to the basolateral compartment was monitored. Transport to the basolateral compartment is the difference between the total ^{125}I transport and non-precipitable radioactivity (free ^{125}I) in the basolateral compartment. An opposite trend was observed when compared to the cell association. Native HDL was transported to the basolateral compartment the most

Figure 13: Association of ^{125}I-apoA-I, ^{125}I-HDL and ^{125}I-rHDL with Polarized HKC-8 Cells

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were incubated with ^{125}I-apoA-I, ^{125}I-HDL or ^{125}I-rHDL particles (300 μg protein/ml) added to the apical compartment at 37°C for various times. The cell lysates were counted to determine total cell associated radioactivity. The rHDL particles (See Table 3 for compositions) were prepared by co-sonification. The data are presented as the mean ± SD of triplicate determinations. A one-way ANOVA was performed to determine statistical significance of difference relative to HDL values (*$p<0.001$).


Cell association
(µg protein/mg cell protein)
16
14
12
10
8
6
4
2
0
HDL
ApoA-I
rHDL-lipid
rHDL-CE
rHDL-apo
*
*
*
*
0
1
2
3
4
5
6
Time (h)

when compared to all other ligands (Fig. 14). ApoA-I and rHDL-apo were observed to have an increased transport to the basolateral compartment when compared to rHDL-lipid and rHDL-CE.

Protein-free radioactivity was monitored by precipitation in 25% TCA in the media in the same experiment. The panels show the protein-free radioactivity for apical and the basolateral compartments as a percentage of total cpm added to the wells for the 6 hour time point (Fig. 15). Native HDL in both apical and basolateral compartments appear to have a maximum of ~5% non-precipitable radioactivity compared to the other ligands. ApoA-I has the lowest non-precipitable radioactivity (1-2%). A similar trend in amounts of non-precipitable radioactivity was observed between the apical and basolateral compartments. Considering that ~ 5% of the non-precipitable radioactivity is background (t=0 free ^{125}I), there appears to be almost no degradative products in the apical and basolateral compartments. This is also supported by the Western blot data. We found no evidence of apoA-I fragments in over-exposed Western blots for apoA-I. To confirm no degradation, future studies can be performed using immunoprecipitation and TCA precipitation on the cell lysates. Similarly, studies suggested that human HDL apoA-I (in contrast to rat HDL apoA-I) is minimally degraded in the kidneys (97). In contrast, nephron microperfusion studies showed that ^{125}I-HDL$_3$ is lysosomally degraded within the rabbit proximal tubule cells (104).

Pulse-Loading Assay

We performed distinct pulse-loading experiments to resolve the transcellular transport of HDL. Polarized HKC-8 cells were pulse-loaded with ^{125}I-HDL (300 μg protein/ml) for one hour. After this incubation, the medium was removed from both

Figure 14: Transport of ^{125}I-native HDL, ^{125}I-rHDL and ^{125}I-apoA-I to the Basolateral Compartment

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were incubated with ^{125}I-apoA-I, ^{125}I-HDL or ^{125}I-rHDL particles (300 μg protein/ml) added to the apical compartment at 37°C for 6 hours. Following the incubation, the medium was removed from both compartments and counted to determine total radioactivity per compartment. An aliquot of the media at the 6 hour time point was analyzed for degradation products by precipitation in 25% TCA. Transport to the basolateral compartment is determined from the difference between the total transport and non-precipitable radioactivity in the basolateral compartment. The rHDL particles (See Table 3 for compositions) were prepared by co-sonification. The data are presented as the mean ± SD of triplicate determinations. A one-way ANOVA was performed to determine statistical significance of difference relative to HDL values (*$p<0.001$).

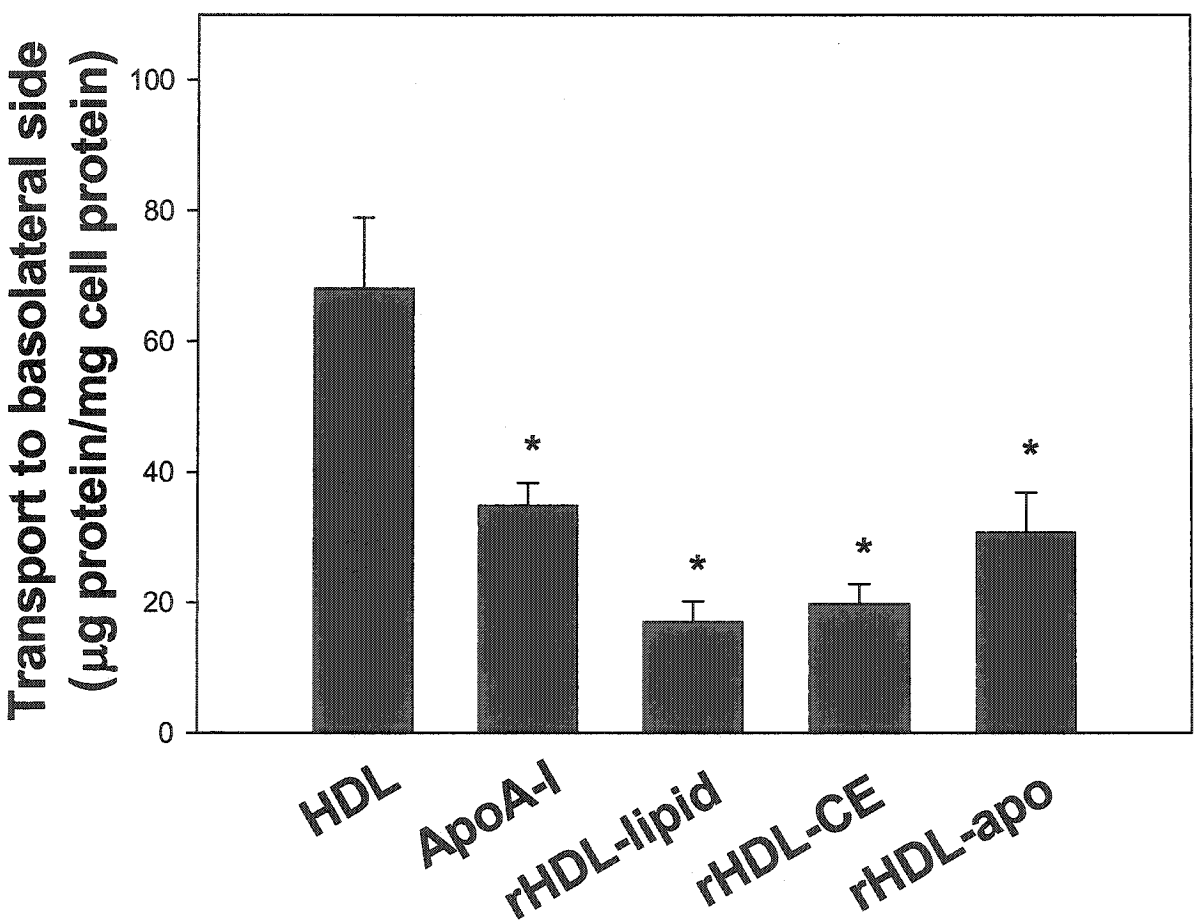
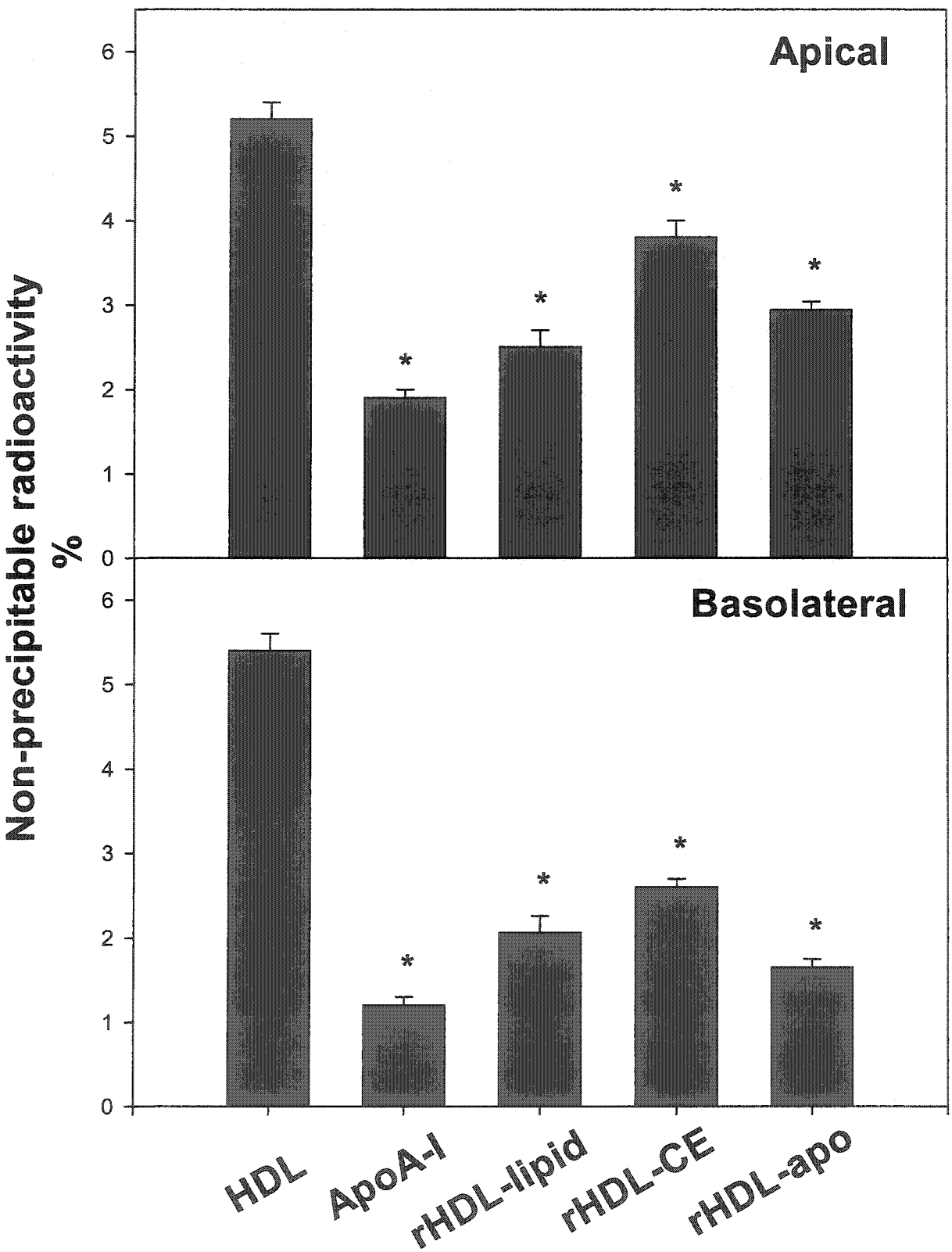

Transport to basolateral side
(μg protein/mg cell protein)
100
80
60
40
20
0
*
*
*
*
HDL
ApoA-I
rHDL-lipid
rHDL-CE
rHDL-apo

Figure 15: Protein-Free Radioactivity of ^{125}I-native HDL, ^{125}I-rHDL and ^{125}I-apoA-I in Polarized HKC-8 Cells

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were incubated with ^{125}I-apoA-I, ^{125}I-HDL or ^{125}I-rHDL particles (300 μg protein/ml) added to the apical compartment at 37°C for 6 hours. Following the incubation, the medium was removed from both compartments and counted to determine total radioactivity per compartment. An aliquot of the media at the 6 hour time point was analyzed for protein-free radioactivity by precipitation in 25% TCA. The panels show the protein-free radioactivity at 6 h for apical and the basolateral compartments as a percentage of total cpms added at t=0. The rHDL particles (See Table 3 for compositions) were prepared by co-sonification.The data are presented as the mean ± SD of triplicate determinations. The data is representative of three experiments. A one-way ANOVA was performed to determine statistical significance of difference relative to HDL values (*$p<0.001$).


Apical
Basolateral
Non-precipitable radioactivity
%
0
1
2
3
4
5
6
*
HDL
ApoA-I
rHDL-lipid
rHDL-CE
rHDL-apo

compartments. Ligand-free medium was added to the compartments and the cells were incubated at 37°C for various times. The cell lysates were counted to determine total cell associated radioactivity (Fig. 16). The t=0 time point is the ligand-cell association after the one hour pulse. We observed that HDL-protein cell associated decreases progressively and plateaus after 2 hours at about 25% of the t=0 value (Fig. 16). Hammad *et al.* observed internalization of ^{125}I-labeled HDL in F9 cells that were differentiated to yolk-sac endoderm-like cells (92). In the same experiment, the transport of labeled HDL to the basolateral compartment was monitored (Fig. 17). We observed that labeled HDL protein is secreted both to the apical and basolateral compartment in pulse-loading experiments. The study showed that HDL protein is secreted ~2 times more to the basolateral compartment when compared to the apical compartment. Maximum secretion appears to occur at the 2 hour time point followed by a loss of radioactivity with time in both the apical and the basolateral compartments.

SDS-PAGE gels were run under reducing conditions to confirm whether intact apoA-I was secreted to the apical and basolateral compartments. Figure 18 shows that intact apoA-I is secreted from the pulsed HKC-8 cells. A decrease in secretion of apoA-I to the basolateral side over time was observed, similar to the results seen with the radioactivity data (Fig. 17).

Cholesterol and Phospholipid Transport Assay

To track the lipid constituents in HDL, polarized HKC-8 cells were pulse-loaded with [^{3}H]-FC-labeled HDL (300 μg protein/ml). The conditions utilized in this experiment were similar to the pulse-loading experiment described above. In Figure 19, cell

Figure 16: Cell Association of ^{125}I-HDL

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were pulse-loaded with ^{125}I-HDL (300 μg protein/ml) added to the apical compartment at 37°C. After one hour, the medium was removed from both compartments. Ligand-free medium was added to both compartments and incubated at 37°C for various times. The cell lysates were counted to determine total cell-associated radioactivity. The data are presented as the mean ± SD of triplicate determinations. The data is representative of three experiments. A one-way ANOVA was performed to determine statistical significance of difference relative to t=0 values (*$p<0.001$).

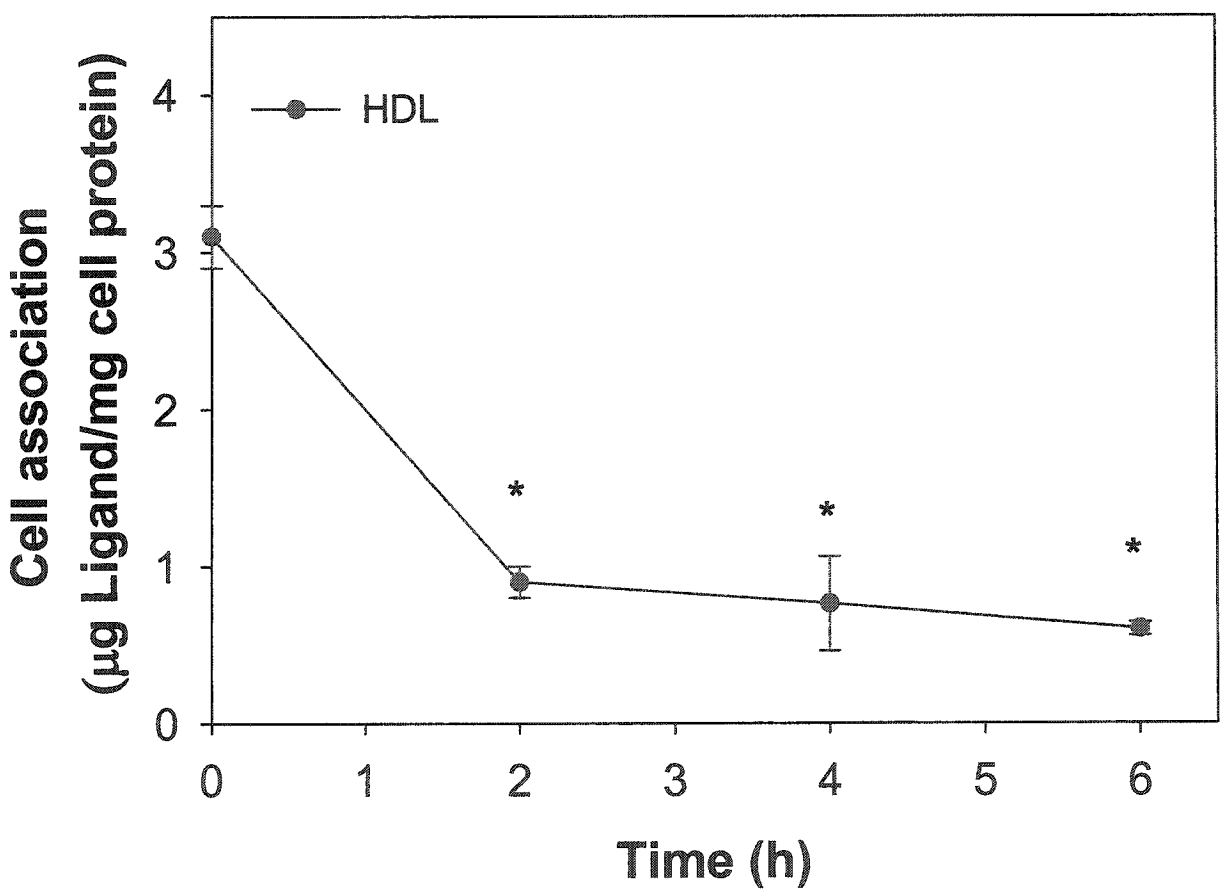

HDL
Cell association
(µg Ligand/mg cell protein)
Time (h)
0
1
2
3
4
5
6
*
*
*

Figure 17: Labeled HDL Protein is Secreted both to the Apical and Basolateral Compartments

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were pulse-loaded with ^{125}I-HDL (300 μg protein/ml) added to the apical compartment at 37°C. After one hour, the medium was removed from both compartments. Ligand-free medium was added to both compartments and incubated at 37°C for various times. Following the incubation, the medium was removed from both compartments and counted to determine total radioactivity per compartment. The data are presented as the mean ± SD of triplicate determinations. The data is representative of three experiments.

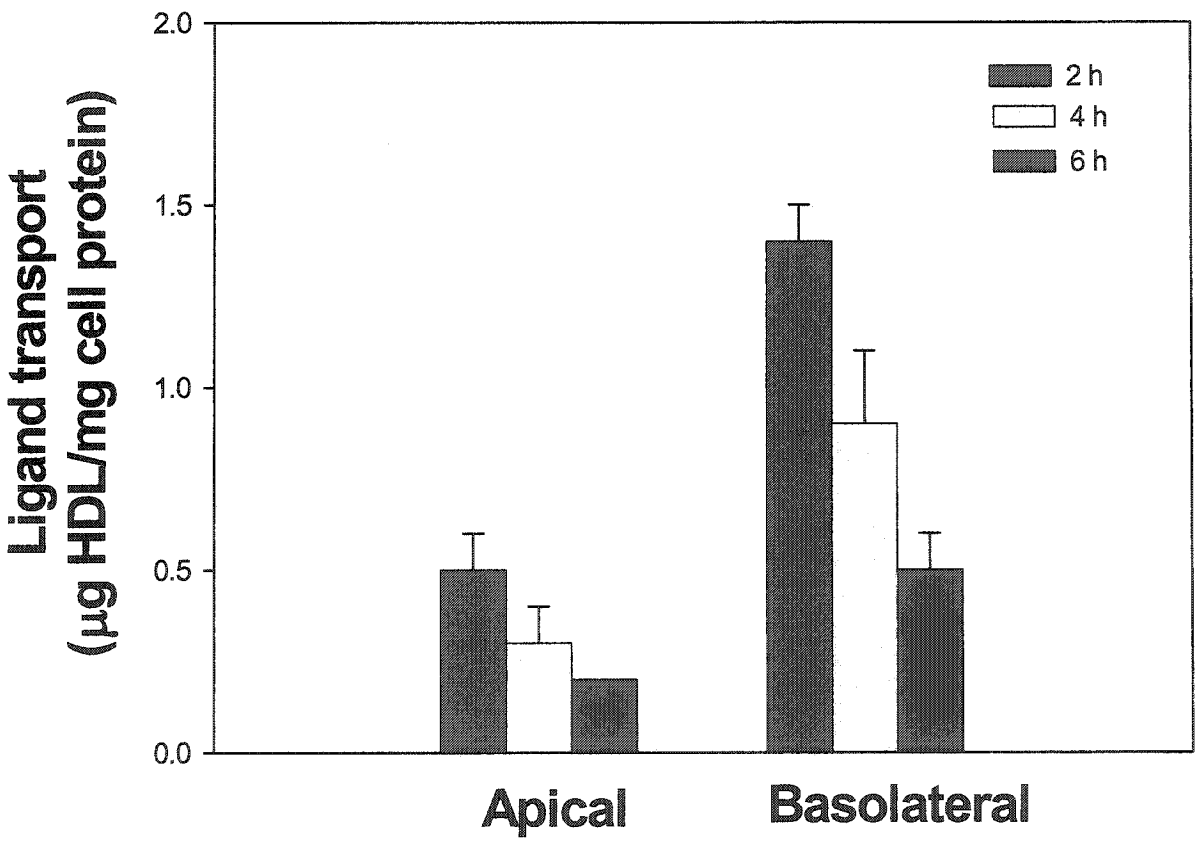

Ligand transport
(µg HDL/mg cell protein)
2.0
1.5
1.0
0.5
0.0
2 h
4 h
6 h
Apical
Basolateral

Figure 18: Intact ApoA-I is Secreted to Apical and Basolateral Compartments

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were pulse-loaded with ^{125}I-HDL (300 μg protein/ml) added to the apical compartment at 37°C. After one hour, the medium was removed from both compartments. Ligand-free medium was added to both compartments and incubated at 37°C for various times. Following the incubation, the medium was removed from both compartments. Western blots for apoA-I were performed on an aliquot of the apical and basolateral media at each time point after it was subjected to 12% SDS-PAGE under reducing conditions. The data is representative of three experiments.

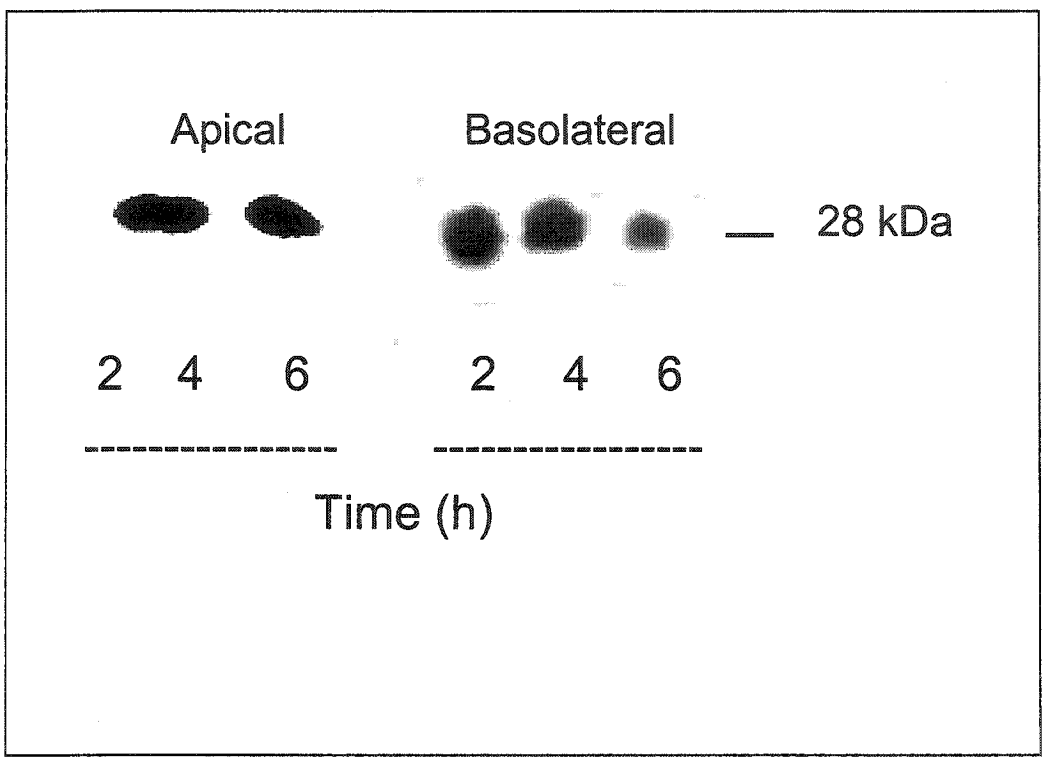
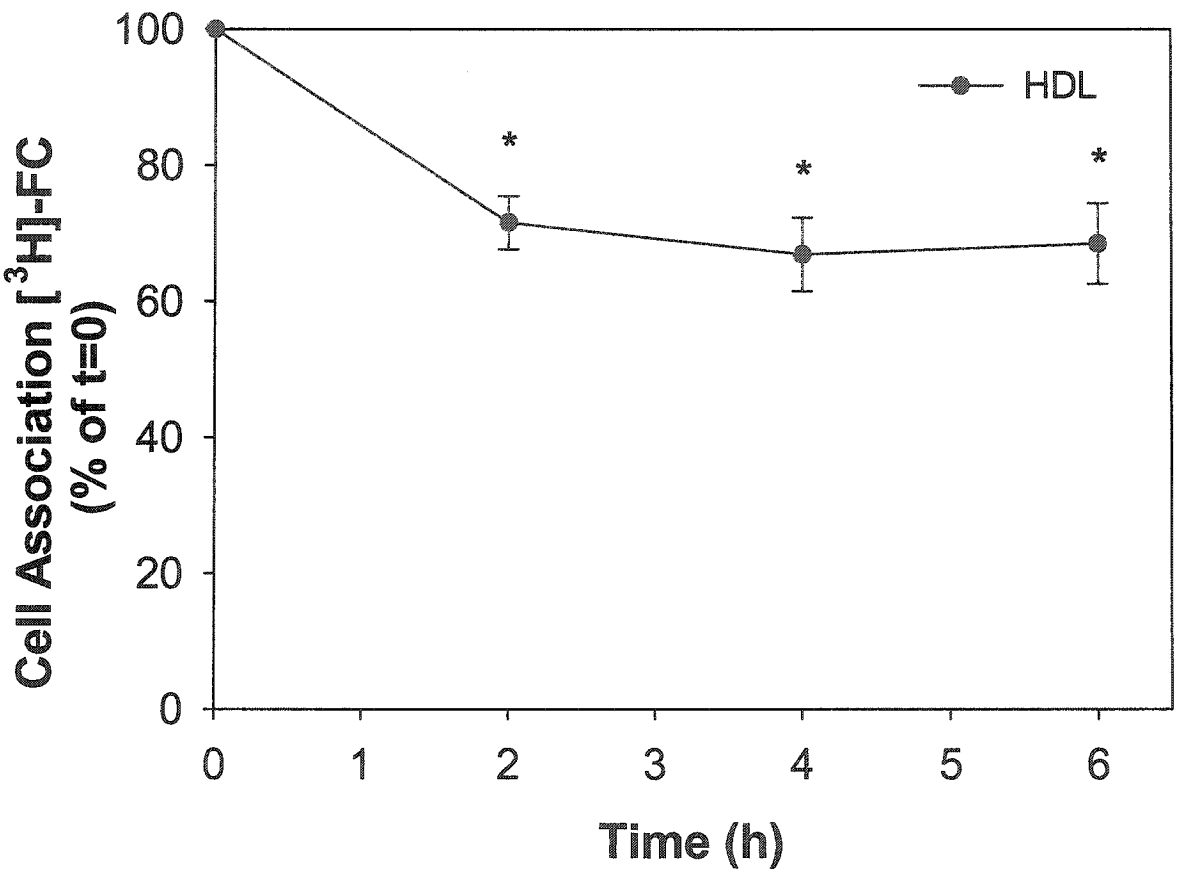

Apical
Basolateral
28 kDa
2 4 6
2 4 6
Time (h)

Figure 19: Cell Association of [^{3}H]-FC

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were pulse-loaded with [^{3}H]-FC-HDL (300 μg protein/ml) added to the apical compartment at 37°C. After one hour, the medium was removed from both compartments. Ligand-free medium was added to both compartments and incubated at 37°C for various time points. The cell lysates were counted to determine total cell associated radioactivity. Cell uptake is presented as a percentage of that measured at t=0. The data are presented as the mean ± SD of triplicate determinations. The data is from one experiment. A one-way ANOVA was performed to determine statistical significance of difference relative to t=0 values (*$p<0.001$).


100
80
60
40
20
0
0
1
2
3
4
5
6
HDL
*
*
*
Cell Association [3H]-FC
(% of t=0)
Time (h)

association is presented as a percentage of the post-loading t=0. There appears to be a progressive decrease in donor HDL-FC cell associated radioactivity to 4 hours. Donor labeled- FC secretion seems to plateau after 2 hours. In Figure 20, the panels show the transport to the apical and basolateral compartment of donor [^{3}H]-FC HDL at the different time-points, as a percentage of t=0. We observed that donor HDL-FC is secreted to both the apical and basolateral compartment. Donor FC is secreted more to the apical side when compared to the basolateral side.

In a similar experiment, polarized HKC-8 cells were pulse-loaded with [^{3}H]-DPPC-labeled HDL (300 μg protein/ml). Cell association is presented as a percentage of the post-loading t=0. We observed a decrease in cell association of donor HDL-PC with time (Fig. 21). In Figure 22, the panels show the transport of donor [^{3}H]-DPPC-HDL to the apical and basolateral compartment at the different time points, as a percentage of the post-loading t=0. We observed that PC is secreted to both the apical and basolateral compartments. PC appears to be transported to both the apical and basolateral compartment to the same extent at the 2 hour time point. There appears to be an increase in secretion of PC to the apical compartment when compared to the basolateral compartment at the 6 hour time point.

From our experimental data, we compared the transport and secretion of different HDL constituents (Table 4). We observed that ~70% of HDL-protein was secreted, while only ~10% of HDL cholesterol and HDL-PL were secreted from the cells. While protein transport to the basolateral side was 3 times that of the apical, more lipid appeared to be secreted to the apical side. Almost 50% of the HDL protein was secreted from the basolateral side, but very little of the donor lipid accompanied the protein.

Table 4: HDL Constituent Secretion to Apical and Basolateral Sides

HDL Constituents	% Transport *	
	Apical	Basolateral
Protein	17	47
Cholesterol	8	3
Phospholipid	6	6

*Transport/secretion was determined as a percentage of post-load, t=0 values.

Phospholipid Enriched Ligand Transport Assay

The effects of PL enrichment of HDL were evaluated with HDL, HDL+PC or HDL+PI (300 μg protein/ml), using pulse-loading assay described above. Western blots for apoA-I were performed on an aliquot of the apical and basolateral media at the 2 hour time point after it was subjected to 12% SDS-PAGE under reducing conditions. In Figure 23A, the SDS Western blot of apical and basolateral media shows that PC enhanced HDL-protein transport to the basolateral media, when compared to HDL and HDL+PI. PI seems to inhibit secretion of HDL to the basolateral side. In contrast, PC enrichment of HDL appeared to inhibit apical re-secretion of HDL protein, while PI stimulated this secretion. Agarose gel electrophoresis of HDL, HDL+PC and HDL+PI used in the experiment showed that PI and PC affects HDL charge (Fig. 23B). Electrokinetic analysis of the gel showed that PI makes HDL more negatively charged, while PC makes HDL less negatively charged (Table 5). Therefore, more positively charged HDL appears to be transcytosed to greater extent than negatively charged HDL.

Table 5: Agarose Electrophoresis Kinetic Analysis

Sample	Migration Distance (cm)	Surface Potential (mV)*
HDL	2.00	-12.2
HDL+PC	1.90	-11.7
HDL+PI	2.50	-14.6

*Lipoprotein surface charge properties were determined from electrokinetic analysis of agarose electrophoretic profiles as described by Sparks and Phillips (250).

Figure 20: Secretion of [^{3}H]-FC from HKC-8 Cells

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were pulsed with [^{3}H]-FC-HDL (300 μg protein/ml) added to the apical compartment at 37°C. After one hour, the medium was removed from both compartments. Ligand-free medium was added to both compartments and incubated at 37°C for various times. Following the incubation, the medium was removed from both compartments and counted to determine total radioactivity per compartment. The data are presented as the mean ± SD of triplicate determinations. The data is from one experiment.

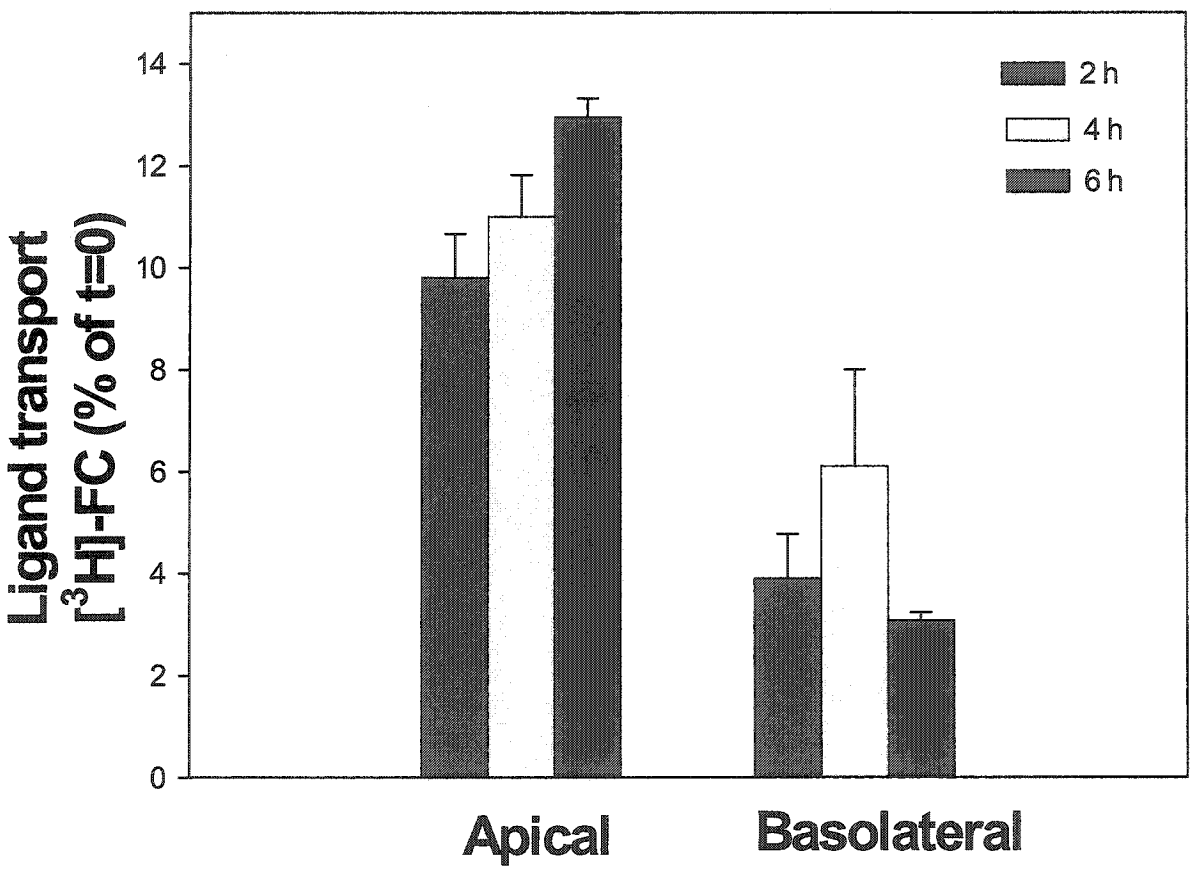

Ligand transport
[3H]-FC (% of t=0)
14
12
10
8
6
4
2
0
2 h
4 h
6 h
Apical
Basolateral

Figure 21: Cell Association of [^{3}H]-DPPC in HKC-8 Cells

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were pulsed with [^{3}H]-DPPC-HDL (300 μg protein/ml) added to the apical compartment at 37°C. After one hour, the medium was removed from both compartments. Ligand-free medium was added to both compartments and incubated at 37°C for various times. The cell lysates were counted to determine total cell-associated radioactivity. The data are presented as the mean ± SD of triplicate determinations. Cell uptake is presented as a percentage of that measured at t=0. The data is from one experiment. A one-way ANOVA was performed to determine statistical significance of difference relative to t=0 values (*$p<0.001$).

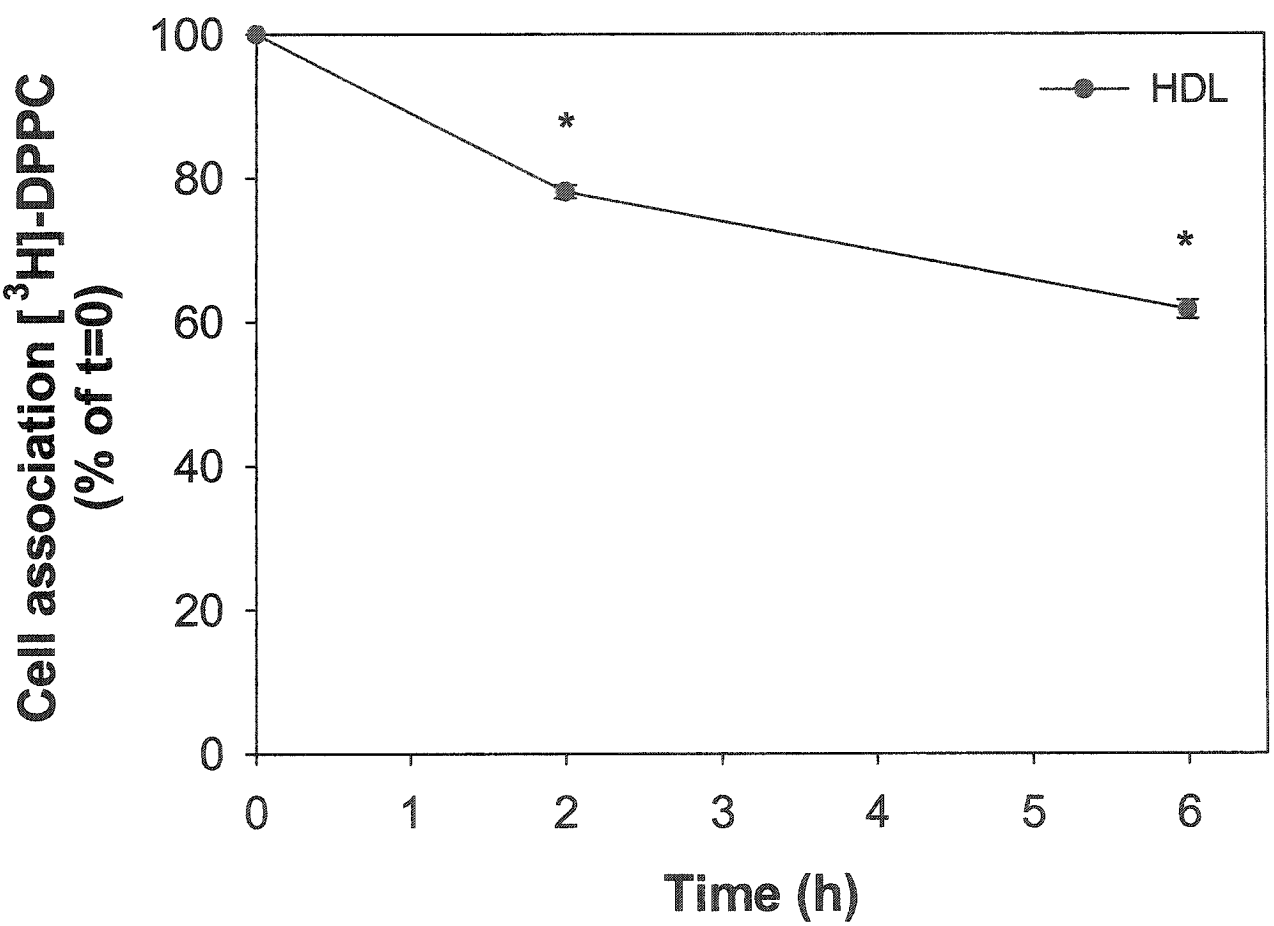

Cell association [^{3}H]-DPPC
(% of t=0)
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (h)
HDL
*
*

Figure 22: Secretion of [^{3}H]-DPPC from HKC-8 Cells

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were pulsed with [^{3}H]-DPPC-HDL (300 μg protein/ml) added to the apical compartment at 37°C. After one hour, the medium was removed from both compartments. Ligand-free medium was added to both compartments and incubated at 37°C for various times. Following the incubation, the medium was removed from both compartments and counted to determine total radioactivity per compartment. The data are presented as the mean ± SD of triplicate determinations. The data is from one experiment.

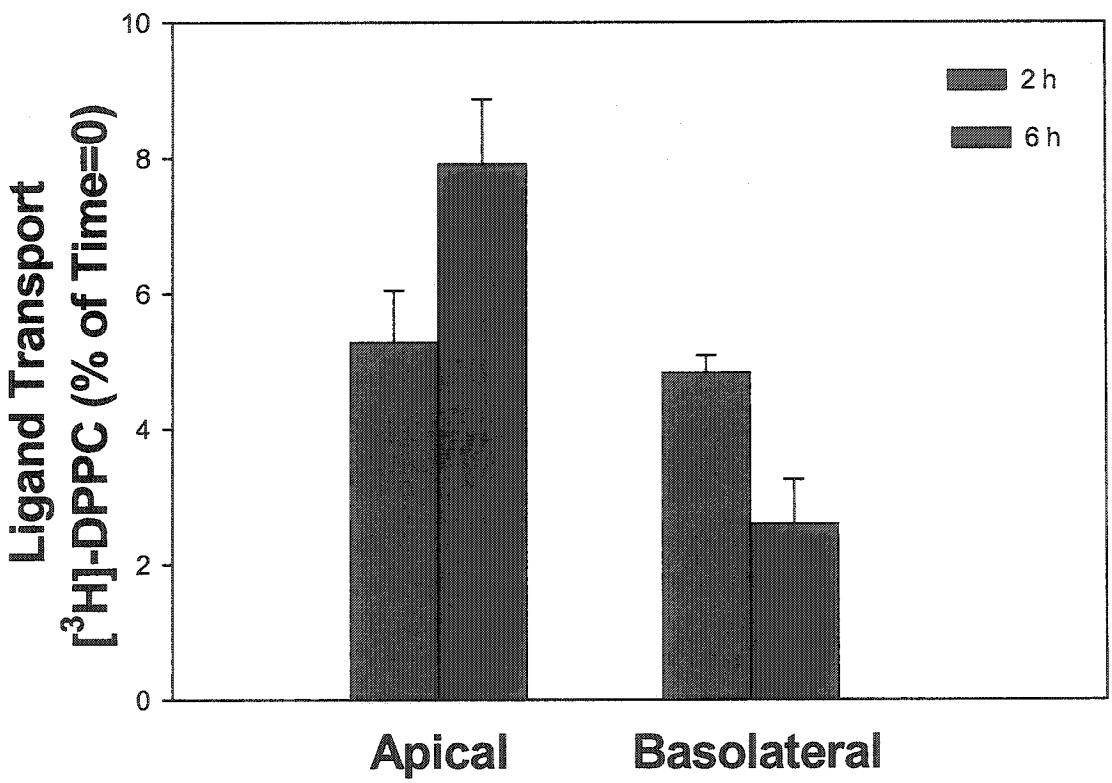
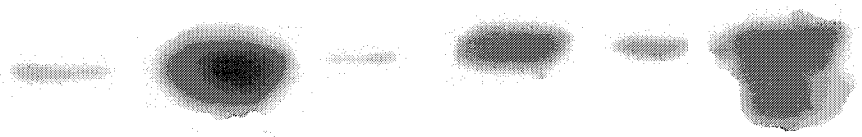

Ligand Transport
[3H]-DPPC (% of Time=0)
10
8
6
4
2
0
2 h
6 h
Apical
Basolateral

Figure 23: HDL Charge Affects ApoA-I Transport

HKC-8 cells cultured on fibronectin-coated Transwell tissue-culture inserts were incubated with [^{3}H]-HDL, [^{3}H]-HDL+PC and [^{3}H]-HDL+PI (300 μg protein/ml) was added to the apical compartment at 37°C. After one hour, the medium was removed from both compartments. Ligand-free medium was added to both compartments and incubated at 37°C for various times. Following the incubation, the medium was removed from both compartments and counted to determine total radioactivity per compartment. Panel A: Western blots for apoA-I were performed on an aliquot of the apical and basolateral media at the 2 hour time point after it was subjected to 12% SDS-PAGE under reducing conditions. Panel B: Lipogel of [^{3}H]-HDL, [^{3}H]-HDL+PC and [^{3}H]-HDL+PI used in the experiment. The data is from one experiment.

A)

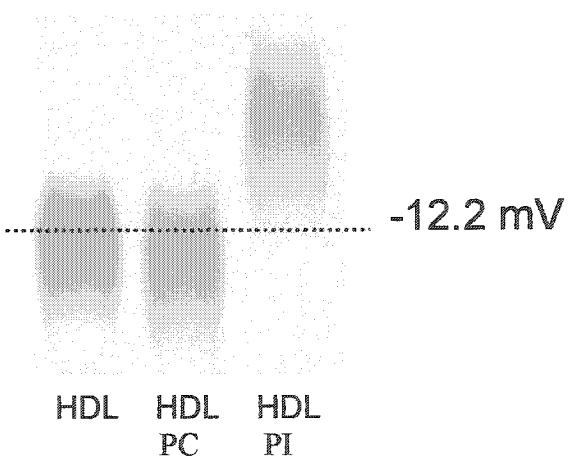


28 kDa

HDL PC PI HDL PC PI

Basolateral Apical

B)



Chapter 4: Discussion

I. Introduction

A strong inverse correlation has been demonstrated between plasma HDL concentrations, their major protein component, apoA-I, and the risk of atherosclerosis. This relationship has generated much interest in elucidating the mechanisms that modulate the concentration of HDL *in vivo* (19-22). The kidney is believed to play an important role in the clearance and re-absorption of filtered lipid-free apoA-I and HDL particles and therefore a characterization of the pathways involved is critical to determine how the levels of HDL in the bloodstream are maintained (22, 96-98, 101). Proximal tubule (PT) epithelial cells of the kidney appear to prevent the loss of HDL proteins in the urine by re-absorbing them from the urinary filtrate (244).

Glass and coworkers observed high rates of apoA-I renal uptake when they studied the tissue sites of degradation of apoA-I (85). Their studies suggested that the high uptake capacity of the kidneys was due to glomerular filtration and tubular reabsorption of free apoA-I. The importance of the kidneys in apoA-I catabolism has been further highlighted by observations from studies of the metabolic turnover of serum HDL apolipoproteins in rats (95). Results from this study showed that a partial hepatectomy had no effect on the fractional turnover rate of HDL apolipoproteins and suggested that HDL may be catabolised in the kidneys. Degradation of HDL apolipoproteins in the kidney appears to be the rate-limiting step in overall HDL apolipoprotein catabolism (95). Braschi and co workers re-evaluated the kinetics of apoA-I and HDL in rabbits after a unilateral nephrectomy (22). Removal of one kidney was associated with a 40-50% reduction in creatinine clearance rates and a 34% decrease in the clearance rate of apoA-I and reconstituted rHDL particles. In contrast, the

clearance of ^{125}I-labeled molecules was much less affected by the removal of a kidney. The data showed that the kidneys are responsible for most (70%) of the catabolism of apoA-I and HDL *in vivo*, while ^{125}I-labeled apoA-I and HDL are rapidly catabolized by different tissues. Thus, the kidneys appear to be a major site for HDL catabolism *in vivo* (22).

The objective of this investigation was to characterize the factors that control the reabsorptive salvage of HDL in the kidney. We explored the role that the proximal tubule cells play in the intracellular metabolism of HDL and determined how the biophysical properties of this lipoprotein affect the reabsorption/catabolism of apoA-I and HDL in these cells. This work has resolved how HDL composition and structure regulates the intracellular metabolism of HDL in polarized proximal tubule cells.

II. *In vivo* Studies

While a number of studies support the view that radioactively-labeled HDL apolipoproteins can be filtered by the kidney glomerulus and can be found in the kidney cortex, some work suggests that the kidneys are also capable of processing whole HDL molecules (100, 104). Previous studies in our laboratory have evaluated the transport of HDL particles through the kidneys *in vivo* and have confirmed this view. Perfusion of a rabbit renal artery with a [^{3}H]-CE and ^{125}I-protein labeled HDL particle showed that the kidneys retained 13% of the [^{3}H]-CE and 32% ^{125}I-labeled protein from the HDL perfusate (244). The ^{125}I protein recovery in the kidneys appears to be in agreement with the literature and shows that the kidneys are responsible for a significant uptake of ^{125}I-apoA-I from the blood (16, 19-21, 85). In addition, the study showed that the kidneys are

capable of retaining both lipid and apoA-I from the blood stream, which suggests that whole HDL particles are filtered through the glomerulus.

Initial studies in our laboratory also performed electron microscopic analysis of kidney tissues after perfusion with apoA-I and rHDL particles. The rHDL particles used were composed of POPC and apoA-I (244). Immunochemical detection of apoA-I was accomplished with a monoclonal antibody (5F6) coupled to colloidal gold. With this antibody, both lipid-free apoA-I and rHDL-apoA-I were only found within the proximal tubule cells of the renal cortex. The results of this study showed that upon filtration, the filtered ligand is re-absorbed from the tubular lumen by the PCT cells of the renal cortex. Both ligands were detected within the basal lamina of the proximal tubule cells.

In summary, previous studies in this and other laboratories have shown that both the protein and lipid components of HDL particles can be found in the rabbit renal cortex and suggest that the rabbit kidneys are involved in the filtration of lipid-free apoA-I and of small, whole HDL particles. The cells of the renal cortex appear to be capable of luminal uptake of the ligands, and of a subsequent delivery of the ligands to the basement membrane where they could be available for re-entry into the circulation.

While previous *in vivo* data provide evidence for processing of HDL by the kidneys, they do not provide details about the mechanisms involved in such processing. To characterize the cellular metabolic pathways that may underlie the findings of the *in vivo* studies, we chose to utilize a cell culture system, using HKC-8 cells, an immortalized human renal tubule epithelial cell line (190).

Although several kidney-cell lines have been established, each model system appears to have some limitations. Available cell lines may only resemble particular

kidney cell types to a limited extent and only a limited number of established kidney cell lines are available. Consequently, culture systems are needed that closely resemble cells in the different nephron segments. Cell lines of renal origin have been utilized previously; Madin-Darby Canine Kidney (MDCK) cells, pig kidney (LLC-PK) cells, ADPKD (autosomal dominant polycystic kidney disease) cells and baby Syrian hamster kidney (BHK) cells (170, 191-198). The major deficiency of these cell lines is that the exact site of origin of the cells within the nephron is largely unknown. Primary cultures have been established and used in other studies (251-255). The ultrastructure of cultured proximal tubule cells can differ in many respects from the original proximal tubule cell. Unfortunately, these cultured cells do not show the typical brush border of proximal tubule cells with densely packed microvilli of uniform dimensions. Cultured cells usually have only rudimentary projections on the apical surface (251, 254, 255). The ultra structure of the vacuolar apparatus in primary cultures is in principle the same as in genuine proximal tubule cells although less developed with respect to invaginations, vesicles and vacuoles, lysosomes, and dense apical tubules. In LLC-PK cells, the intercellular spaces are narrow and the membranes show interdigitations (256, 257), although not very extensive. The vacuolar apparatus may differ considerably in established cell lines (124).

Therefore, an optimized and differentiated human renal epithelial cell line with extended *in vitro* growth potential would provide an alternative model system to primary culture or other available non-human mammalian kidney cell lines. For this purpose, human renal tubule epithelial cells were isolated from normal kidney cortex and exposed in culture to a hybrid immortalizing virus, adenovirus 12-SV40 (190). These

immortalized human renal PT epithelial cells, the HKC-8, express differentiated features of proximal tubular cells and are comparable, and in some cases, superior to established cell lines (LLC-RK1, OK, HK-2) as well as to human PT cells in prolonged primary culture. The cell line has been shown to maintain normal expression of tubule markers and biochemical properties and to display a normal epithelial monolayer morphology with a well developed brush border for prolonged periods (190). Polarized HKC-8 cells are a valuable tool to explore the molecular mechanism of polarized membrane trafficking pathways in the human proximal tubules.

III. *In vitro* Experiments with Non-Polarized HKC-8 Cells

Our laboratory has conducted initial studies with HKC-8 cells grown on conventional plates in a non-polarized state. Immunofluorescence studies with confluent non-polarized HKC-8 cells showed that these proximal tubule cells can readily bind and take up HDL particles (244). Intracellular localization of fluorescently-labeled HDL showed an accumulation of the lipoprotein in vesicles in the perinuclear region after 1 h. Peterson *et al.* proposed that the vesicles that accumulate ^{125}I-HDL upon perfusion may be apical endocytic vesicles, vacuoles and lysosome-like dense bodies; fundamental components of the endocytic apparatus of the proximal tubule cells (104). HKC-8 cells therefore appear to be ideal model for our studies as they are capable of binding, internalization and intracellular metabolism of HDL particles.

In order to evaluate the effect of structural components of HDL on its association with human proximal tubule cells, a series of time-course experiments in HKC-8 cells using ^{125}I-labeled HDL, rHDL and apoA-I were performed (244). These studies showed a saturable binding of ^{125}I-ligand to the HKC-8 cell surface over 2 h incubation. Data

obtained from competitive binding assays with HKC-8 cells showed that HDL can efficiently bind to the cells through a saturable and high affinity binding process. Further, these studies showed that the specific binding of native HDL was approximately 5-fold higher than that observed for lipid free apoA-I. In contrast, more apoA-I was secreted into and retained in the HKC-8 ECM with lipid-free apoA-I than with native HDL particles (244). These experiments also showed that rHDL prepared from extracted lipids from native HDL bound to the HKC-8 cells to a similar extent as native HDL, while rHDL prepared from pure PL bound 7-fold less. Inclusion of a variety of different lipids into the rHDL complex had little effect on cell association, but inclusion of CE into the lipoprotein enhanced binding ~2-fold. Therefore, the binding/association of HDL with proximal tubule cells appears to be affected by the CE composition of the lipoprotein (244).

In summary, data from the non-polarized HKC-8 cells showed that the human HKC-8 proximal tubule cell line is capable of binding and internalizing HDL particles. Cell incubations show a saturable, specific binding of ^{125}I-HDL, to the cell surface in a manner suggestive of a receptor-mediated association. The lipid composition of the HDL particles appears to play a major role in this specific association, as some components, notably CE, of the HDL-lipid fraction markedly enhance the association of HDL to these HKC-8 cells (244).

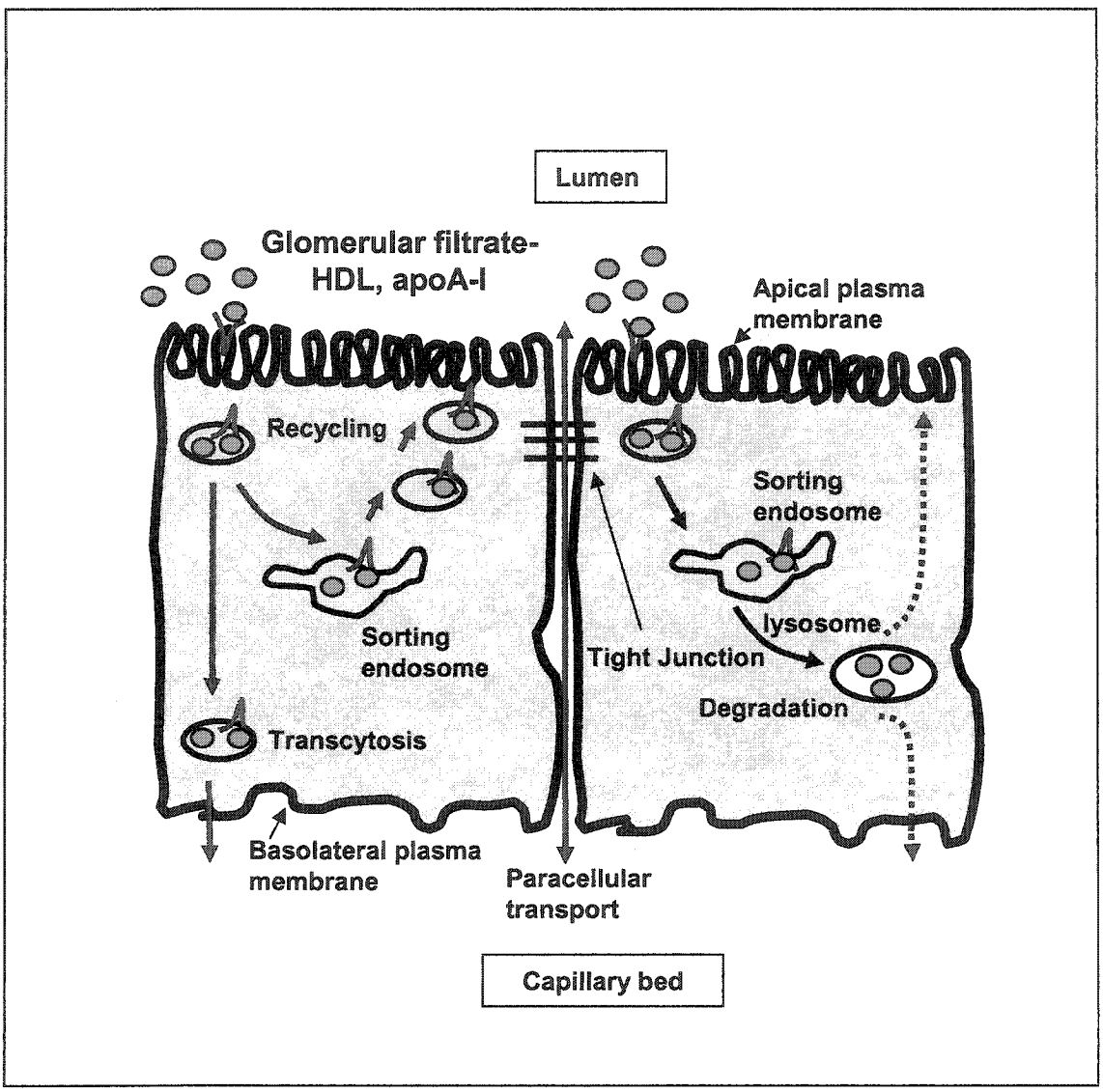
IV. *In vitro* Experiments with Polarized HKC-8 Cells

The focus of the present work was to study the intracellular metabolism of HDL in polarized HKC-8 cells grown on Transwell tissue-culture inserts. We observed significant differences in the cellular metabolism of HDL when we compared

experiments using conventional plates to experiments with polarized cell cultures. Polarity is a fundamental property of cells in living organisms (151-154). Epithelial cells, exhibit cell-surface polarity, which means that their plasma membrane is divided into morphologically distinct apical and basal regions separated by cell junctions (Fig. 24). They are exposed to different environments, which have a characteristic protein and lipid composition. The basolateral plasma-membrane domain faces the blood circulation and adjacent cells, whereas the apical domain is in contact with the external environment, such as the bile canaliculus in hepatocytes or the lumen in renal and intestinal epithelial cells (155-166). These domains are separated by tight junctions to prevent intermixing of membrane components (Fig. 24). To generate and maintain this structural polarity, cells need mechanisms to specifically target newly synthesized proteins and lipids to the correct surface (162). Biochemically differentiated microdomains that correspond to structures involved in endocytosis and transcytosis are generated on the endothelial cell surface due to difference in surface charge. The preferential distribution of specific receptors on the luminal or abluminal plasmalemma, play a critical role in the uptake, intracellular routing, and direction of transport of their respective ligands (167). To obtain a detailed understanding of polarized membrane trafficking, it is important to characterize how the transport of proteins and lipids are regulated (155). While our previous *in vitro* data using non-polarized cells provided evidence that the human HKC-8 proximal tubule cells are capable of binding and internalizing HDL particles, they do not provide details about the mechanisms involved in such processing. Well characterized polarized *in vitro* model systems may be of value

Figure 24: Potential Transport Pathways across an Epithelial Monolayer

Lipoprotein particles can cross an epithelial cell barrier by being transported through the cell (transcytotic transport) or by moving between adjacent cells (paracellular transport). Paracellular transport is passive and results from diffusion, electrodiffusion, or osmosis down the gradients created by transcellular mechanisms. The major barrier in the paracellular route is the tight junction, which is regulated and varies among epithelia in porosity and ion selectivity. Transcytotic transport occurs after lipoproteins are endocytosed through receptor and non-receptor mediated uptake pathways. The lipoprotein then enters an endosomal compartment where cargo sorting occurs. Lipoproteins can be transcytosed to the opposite pole, the basolateral membrane, or retroendocytosed (recycled) back to the apical surface. Lipoproteins can also be transported to the lysosome for degradation. With proximal tubule epithelial cells, HDL appears to be transported both by transcytotic and paracellular mechanisms.


Lumen
Glomerular filtrate-
HDL, apoA-I
Apical plasma
membrane
Recycling
Sorting
endosome
Sorting
endosome
lysosome
Tight Junction
Degradation
Transcytosis
Basolateral plasma
membrane
Paracellular
transport
Capillary bed

to simulate the *in vivo* model system and to explore the molecular mechanism of polarized membrane trafficking pathways.

An important feature of epithelial cells grown in a polarized setting is their ability to limit paracellular diffusion. Such paracellular leakage could interfere with the interpretation of the data and with differentiation of paracellular and transcytotic transport (Figure 24). Paracellular transport is passive and results from diffusion, electrodiffusion, or osmosis down the gradients created by transcellular mechanisms (162, 209-212). Individual cells in an epithelial sheet are attached to each other by a set of contacts called the epithelial junctional complex (213-215). The tight junction is the major physical structure defining the specific properties of the paracellular barrier (Fig. 24). Tight junctions, or zonula occludens, create a paracellular barrier for passive diffusion so that the electrochemical gradient of the epithelium can be maintained. Disruption or interference of the epithelial tight junctions may therefore contribute to paracellular leakage (258). They are also thought to be important for the maintenance of biochemically distinct cell surface domains by forming an intramembrane fence that restricts the diffusion of lipids in the outer leaflet of the plasma membrane (162, 211, 216, 259).

Previous studies in our laboratory have utilized several different methods to determine if the HKC-8 cells form a monolayer diffusion barrier and whether we could track the active processing of HDL by the HKC-8 cells and differentiate this from passive paracellular leakage. We measured transepithelial electrical resistance (TER) using Millicell-ERS (Electrical Resistance System), biochemical methods ([^{14}C]-inulin, [^{3}H]-

mannitol) and spectroscopic methods (phenol red transepithelial) to ensure that the HKC-8 cells form a tight monolayer in the polarized system. We observed that the cells formed a tightly sealed monolayer that is virtually impenetratable to small molecules (244). We also routinely assessed [^{14}C]-inulin diffusion across the cell monolayer in the experiments described and similarly showed that the HKC-8 cells cultured on fibronectin-coated permeable filters provide a relatively impermeable diffusion barrier to passive paracellular diffusion.

1. **Cell Association**

Breznan *et al.* (244) observed a reduced association of lipid-free ^{125}I-apoA-I with HKC-8 cells in comparison to that for ^{125}I-native HDL (244). Pure ^{125}I-apoA-I has also been shown to compete less effectively for HDL binding sites than ^{125}I-HDL$_3$ in human enterocytes that display numerous structural and functional similarities to renal epithelia (245). In contrast, our studies with polarized HKC-8 cells show that native HDL associates with the cells to a lesser extent than that observed for apoA-I (Fig. 13). This suggests that polarity may dramatically affect cellular function. Epithelial cells can undergo extensive morphological and biochemical re-modeling as a result of a decrease in the proliferation and an increase in the differentiation of the cells when grown in a setting enabling cell polarity in comparison to the solid surface culture (158). This suggests that even though epithelial cell culture by solid surface *in vitro* techniques (non-polarized) is common place for studying cellular metabolism, metabolic results from polarized cells may differ significantly.

While apoA-I may be important for the initial association of HDL with HDL receptors (86), the HDL lipid composition appears to be also important in determining the

affinity of this interaction (244). Our studies with the different HDL subclasses showed that VHDL and HDL_2 could associate more with the polarized HKC-8 cells when compared to HDL_3 (Fig. 6). This data suggests that lipoprotein composition and/or size may influence cell association of lipoprotein particles. Studies have shown that alterations in the composition of lipoprotein particles can affect their charge and structural properties and that these characteristics will influence lipoprotein remodeling and plasma lipid metabolism (22, 158, 260). Furthermore, numerous studies show that HDL-lipid composition has major effects on the conformation of apoA-I and the stability and charge of the apoA-I containing HDL particle (50, 261-263). Previous experiments in our laboratory using non-polarized HKC-8 cells have shown that ^{125}I-rHDL reconstituted with CE showed a 2-fold increase in specific cell association when compared to that of ^{125}I-rHDL containing no CE (244). Reconstitution of rHDL particles with specific lipids; DG, TG, Sphingomyelin (SM), PI and FC did not enhance rHDL specific association with the cells to levels comparable to the control HDL particles (244). In studies with polarized HKC-8 cells we show that all rHDL particles appeared to bind/associate with the cells to a greater extent than HDL at all time points (Fig. 13). This is similar to that observed with some of the different HDL subfractions. However, the internalization and transport of these lipoproteins through the HKC-8 cells appeared to be very similar. This may suggest that the specific binding and uptake pathways are comparable for the different ligands, but that some kinds of HDL may exhibit a very high non-specific association with the cells. This is consistent with the study of Breznan *et al.*, which showed that rHDL exhibit a much higher non-specific association with this same cell system than that observed for native HDL (244). The consequence of a very high non-

specific association is unclear. Studies have previously shown that rHDL particles exhibit a decreased negative surface charge and stability relative to that observed for native HDL (14). Previous work has identified a relationship between HDL surface charge and stability and the clearance of this lipoprotein from the plasma (14). Taken together with the view that the kidneys are important to HDL clearance *in vivo* (22), the data may suggest that HDL composition affects the renal handling of this lipoprotein.

Several laboratories have demonstrated ^{125}I-HDL binding to the renal cortex in different species, including those of rat, porcine and human origin (97, 98, 103). Van Tol and colleagues have identified a low-affinity non-saturating and also a high affinity saturating rat HDL binding to partially purified rat kidney membranes or kidney homogenates (97). The binding also appeared to be species-specific, as unlabeled human HDL was a poor competitor for the rat lipoproteins (97). A benefit to our experiments is that they were done with human HDL and in a human proximal tubule cell line. Further competitive binding assays would be required to establish the affinity and specificity of HDL binding to HKC-8 cells or any role that a specific HDL binding protein or receptor may have in this process. Senault and coworkers have described a two component association of porcine ^{125}I-HDL to basolateral membranes of the porcine renal cortex; a high-affinity, lower capacity and a low affinity, higher capacity interaction with ^{125}I-HDL (103). The authors suggested that the low-affinity, higher capacity binding component involved a non-specific lipid-lipid interaction with the membrane, as the interaction was not species specific and was out-competed by an excess of unlabeled LDL (103). Kozyraki *et al.* identified a high-affinity binding of human ^{125}I-HDL to a 280 kDa receptor purified from the human as well as the rabbit renal cortex, cubulin (98). The

interaction was Ca^{2+} ion dependent, apoE independent and required apoA-I, as anti-apoA-I antibodies strongly inhibited HDL uptake. Cubulin appears to be localized exclusively in the apical membranes of the renal cortex (264). Cellular uptake studies of fluorescent PL-labeled HDL or ^{125}I-HDL showed that HDL underwent cubulin-mediated endocytosis, and transport to lysosomes (92, 98). The saturable binding of ^{125}I-HDL to HKC-8 cells observed in our assay may therefore be the result of these unique higher-affinity specific interactions. There is a strong possibility that HDL association seen in our studies may be the result of its binding to cubulin, as the binding was saturable and may involve an endocytic processing of HDL after cell surface interactions with polarized HKC-8 cells.

2. Internalization

To determine whether HDL particles were internalized into polarized HKC-8 cells we utilized a variety of tracking systems. We utilized radioactive lipids and ^{125}I-labeled protein to track the different HDL constituents. We also used a mix of monoclonal antibodies directed against a central domain (5F6) and N-terminus of apoA-I (4H1) to detect and quantify HDL and apoA-I. With each tracking system we observed that HDL and apoA-I were taken up into the polarized HKC-8 cells. This corroborates our early fluorescence studies and shows that HDL is internalized by proximal tubule cells.

Experiments were performed with radioactive labeled lipoproteins to estimate the amounts of cell internalized and the surface bound ligand from total cell uptake of different native HDL subfractions. All three HDL classes appeared to be internalized to the same extent (Fig. 10). To confirm this result and further differentiate internalization from total cell association of the three subfractions of HDL, we designed an experiment

and measured the amount of intracellular apoA-I immunochemically. In this series of experiments we observed significantly more internalization of HDL_3 when compared to the other HDL subfractions (Fig. 11). The result showed that these HDL_3 particles were internalized to a greater extent by HKC-8 cells. The difference in result compared to the tracer studies could be partly attributed to the variations in the techniques used and may also be due to the differences in lipoprotein donors used in these studies. The result suggests that some HDL subclasses may be preferentially taken up by HKC-8 cells. Similarly, Rogler and coworkers observed that intact HDL_3 is bound specifically by polarized Caco-2 cells, leading to subsequent intracellular passage (176). Renal tubular reabsorption and hydrolysis of plasma HDL_3 were studied using rabbit proximal straight nephron segments (104). The segments were microperfused *in vitro* with iodinated HDL_3. Electron microscopic radioautography demonstrated endocytic uptake of ^{125}I-HDL_3 at the luminal membrane of the proximal tubule and movement of grains into lysosome-like dense bodies. Collectively, the data shows that plasma HDL_3 could be preferentially reabsorbed in the proximal nephron by a mechanism involving endocytosis at the luminal membrane, followed by proteolysis in the lysosomes (104). Klinger *et al.* attempted to visualize how Caco-2 cells process HDL particles using an immunocytochemical post-embedding method in polarized intestinal Caco-2 cells (177). They observed that HDL_3 is internalized and subsequently processed in an endosomal pathway in Caco-2 cells (177). There is a strong possibility that internalization of HDL_3 seen in our experiments could also be due to endocytic uptake by HKC-8 cells. Further studies need to be conducted to establish the pathways involved in endocytic internalization.

3. Degradation

The kidney is believed to play an important role in the re-absorption of filtered lipid-free apoA-I and HDL particles (22, 96-98, 101). Proximal tubule epithelial cells of the kidney appear to prevent the loss of these proteins in the urine by re-absorbing them from the urinary filtrate (244). If these proteins are not re-absorbed by the cells, they may instead be degraded and lost (Fig. 24). Since minimal amounts of apoA-I or apoA-I fragments are found in the urine, the kidney appears to play a role in both the re-absorptive salvage of apoA-I and in its degradation. Whether this degradation occurs in the proximal tubule cell is a question that we addressed directly.

Early studies with HKC-8 cells showed that ^{125}I-HDL, ^{125}I-apoA-I and ^{125}I-rHDL particles underwent very minimal degradation in experiments with HKC-8 cells grown on conventional plates (244). With non-polarized cells we observed less than 2% TCA soluble radioactivity in the cell media, which was comparable to background free ^{125}I values and indicative of little or no labeled protein degradation. Experiments using polarized HKC-8 cells also show almost no degradative products in the apical and basolateral compartments, when evaluated with radioactive tracer liberation or by Western blots (Fig. 15). To confirm no degradation, future studies can be performed using immunoprecipitation and TCA precipitation on the cell lysates. Studies have shown that human HDL apoA-I is degraded to a lesser extent than rat HDL apoA-I in rat kidneys (97). ApoA-I catabolism in the kidneys could be species specific. Bulger and Trump suggested that endocytic vacuoles or deep plasma membrane invaginations could empty their contents into the intercellular space and thereby protein could pass the proximal tubule wall undegraded (149). Studies showed that glucagon (a linear peptide) is

degraded by the brush border hydrolases (124), whereas insulin is taken up by endocytosis (265). These observations could suggest that smaller linear peptides are degraded by the brush border peptidases, whereas larger peptides such as insulin that contain two disulphide bridges are resistant to degradation and instead are taken up by endocytosis (124). Similarly, our observation may suggest that larger lipoproteins (HDL) could be resistant to degradation in PT cells and be taken up by endocytosis. However, the possibility cannot be excluded that larger peptides or proteins are cleaved to a limited degree at the brush border and the larger degradation products then undergo endocytosis (124). Proteases in PT cells can degrade ^{125}I-HDL and generate radiolabeled breakdown products (i.e., polypeptides and monoiodotyrosine) which may diffuse out of membrane bound organelles because of their small size and accumulate within the cell cytoplasm or be lost from the cell into the media (266). Significant degradation is associated with leakage of free ^{125}I. Since we observed minimal leakage of tracer, the data suggests that significant amounts of HDL degradative products were not generated in our experiments with polarized HKC-8 cells.

In contrast to our results, nephron microperfusion studies have shown that ^{125}I -HDL_3 is lysosomally degraded within rabbit proximal tubule cells (104). Numerous studies for a variety of proteins using isolated lysosomal fractions, isolated intact lysosomes, renal cortical slices, isolated perfused kidneys demonstrated that proteins that are reabsorbed and transported into lysosomes are degraded to amino acids (124). This lysosomal hydrolysis was also implied from the disappearance of absorbed protein from lysosomes in intact animals (124). Studies have also shown significant levels of lysosomal hydrolysis of ^{125}I -HDL in mouse yolk sac endoderm-like cells expressing

cubulin (98). These studies were performed in animal models using human lipoproteins. Studies analyzed the catabolic sites of human and rat high-density lipoprotein in the rat using the lysosomal cathepsin inhibitor leupeptin (268). Their results showed that radioiodinated rat HDL was catabolized by the kidneys and by the liver. In contrast, radioiodinated human HDL was catabolized almost exclusively in the rat liver. They concluded that in the rat, the catabolic pathways of apoA-I moieties of rat and human HDL are different, indicating that homologous HDL should be used for the investigation of *in vivo* metabolism (268). In addition, studies demonstrate that iodination of tyrosine residues alters the apoA-I molecule in a manner that promotes an accelerated catabolism (22). Our polarized *in vitro* studies were performed using human lipoproteins in a human proximal tubule cell line. Nonetheless, we saw negligible degradation in tracer studies and no degradative fragments in our immunostudies. This result is not surprising, since these cells are indeed a renal cell line that would normally be expected to salvage proteins from the glomerular filtrate. Our results could imply that HDL degradation occurs in the later regions of the tubule. Further *in vivo* and *in vitro* degradation studies need to be performed using various sections of the renal convoluted tubule to clarify this assumption. In addition, we used a transformed cell line (HKC-8) in our experiments, which may also contribute to the variations seen in our results.

Our results may suggest that internalized HDL may be transported to subcellular compartments other than lysosomes for intracellular transport. Studies have shown a significant catabolism of HDL/HDL apolipoproteins in the mouse yolk-sac endoderm-like cells following cubulin-mediated endocytosis (92). They have also shown that chloroquine treatment, which inhibited ^{125}I-HDL degradation, did not lead to an

intracellular accumulation of internalized ^{125}I-HDL. Typically, chloroquine treatment leads to intracellular accumulation of ligands targeted for lysosomal degradation such as LDL (269). It is therefore possible that HDL/HDL apolipoproteins can be trafficked via at least two pathways after cubulin-mediated endocytosis, one that leads to the lysosomal degradation and another that leads out of the cell (e.g. transcytosis) (Fig. 24). This suggested the existence of a second alternative pathway for trafficking of HDL/HDL apolipoproteins which escape degradation in the PCT cells (92). Studies suggest that internalized protein may be transported to subcellular compartments other than lysosomes for transcytosis (124, 229). Our studies show that HDL enters a transcellular pathway in the HKC-8 cells that allows for a rapid transport of the lipoprotein through the cell.

4. Intracellular Transport

In the kidney, plasma proteins filtered through glomeruli are reabsorbed by various routes along the proximal tubules to avoid renal loss of large amounts of proteins (230). Transcytosis and paracellular transport are the major membrane-trafficking pathways used to transport cargo from one side of the cell to the other (162, 169, 202, 203) (Fig. 24). Transcellular transport, the movement of ions and small molecules through a cell, is accomplished by the differential distribution of membrane transporters/carriers on opposite sides of a cell. Paracellular transport, the movement between adjacent cells, is accomplished by regulation of tight junction permeability (162, 209-212) (Fig. 24).

Paracellular Transport

Pulse-loading experiments with ^{125}I-HDL showed that at one hour, 1-2 μg of HDL/mg cell protein is secreted to the basolateral compartment (Fig. 17). The transport

seen in these experiments appears to be primarily due to transcytosis (Fig. 24). Previously, we performed similar experiments using radiolabeled HDL fractions where we incubated the ligand with the cells in the apical media for longer periods. In contrast to the pulse-loading experiments, we observed around 3-5 μg of HDL/mg cell protein was transported to the basolateral side at a comparable time point (Fig. 7). This increase in transport could partly be attributed to paracellular transport (Fig. 24) or simply to differences in the experimental conditions.

Experiments were performed to determine whether HDL is secreted from the basolateral side of the cell as a whole HDL particle or whether it gets remodeled/degraded and resecreted. Non-denaturing western blots of the apical and basolateral media showed that when whole native HDL is added to the apical side of polarized HKC-8 cells, only HDL_3 size particles appear to be secreted from the basolateral side (Fig. 9). In contrast, Figure 12 clearly shows that there is no dramatic remodeling of the lipoproteins, since all three HDL subfractions were secreted from the cell without apparent modification in size. This difference may be partly due to an underexposure of gel shown in Figure 9 and emphasis on the predominant species in the western blot.

Epithelial transport occurs through both transcytosis and paracellular pathways (211). Paracellular transport is passive and results from diffusion, electrodiffusion, or osmosis down the gradients created by transcellular mechanisms (162, 209-212). Thus the paracellular pathway complements transcellular mechanisms by defining the degree and selectivity of back leak for ions and solutes, making an all important tissue-specific

contribution to overall transport. The tight junction is the major physical structure defining the specific properties of the paracellular barrier.

Studies show that in proximal tubules perfused with a high chloride-low bicarbonate solution simulating late proximal tubular fluid, approximately half of NaCl transport is active and transcellular, and half was found to be passive and paracellular (270-272). Most of the results obtained, so far, indicate that transcytosis of albumin, low-density lipoproteins, metaloproteases, and insulin, is performed by cargo-vesicles and their generated channels. The paracellular pathway can be used for water and ions; in postcapillary venules, at the level of which ~30% of junctions are open to a space of 6 nm, small molecules may take this route (162). While apoA-I may be small enough to be transported paracellularly, HDL particles are greater than 6 nm in size and therefore are probably not transported through paracellular routes to any great extent.

Retroendocytosis

It has been generally accepted that the serum proteins are absorbed in the proximal tubules by a process of endocytosis that either delivers the proteins to the lysosomes where degradation occurs or transports them through the cell for salvage (273). Studies suggest that insulin may be handled somewhat differently. In a variety of cell types, including hepatocytes, adipocytes, and endothelial cells, it is now well established that there are alternate transport pathways for insulin, other than the classic lysosomal route. These alternative pathways include trafficking to the nucleus, trafficking across the cell (transcytosis), or trafficking back to the cell surface with exocytosis. This latter route has been referred to as retroendocytosis, and a number of other ligands,

including transferrin, growth hormone, and asialoglycoproteins, utilize this pathway (273) (Fig. 24).

We observed that a significant amount of labeled HDL protein is also secreted to the apical compartment in pulse-loading experiments (Fig. 17). The transport of labeled HDL to the apical compartment seen in the pulse-loading experiments may therefore be due to retroendocytosis (Fig. 24). Western blot analysis confirmed that intact apoA-I and HDL are transported to the apical surface after cell uptake (Fig. 18). This may suggest that HDL can be retroendocytosed to the apical surface and be released into the proximal tubular lumen (Fig. 24) and this recycling of HDL could account for the loss of some HDL in the urine. Studies suggest that the dense apical tubules that are often seen connected to endocytotic vacuoles are responsible for membrane recycling between the endocytic vacuoles and the apical plasma membrane and this view has been substantiated by studies using cationized ferritin (274, 274). Experiments with isolated perfused rabbit proximal tubules at low temperature have shown that gold-labeled particles, coated with insulin and cross-linked to the luminal plasma membrane, first appear in endocytic vesicles and vacuoles and then later in dense apical tubules. This recycling pathway bears some resemblance to the CURL system (compartment of uncoupling of receptor and ligand) in hepatocytes and the recycling suggested for macrophages (124). Macrophages, hepatocytes, and fibroblasts have been shown to internalize lipid-free apolipoproteins, HDL and chylomicron remnants and to then resecrete lipidated apolipoproteins, through a process termed retroendocytosis (275, 276). When HDL particles are internalized by non-polarized hepatocytes, they appear to be trafficked to the endocytic recycling compartment (a transferrin-positive compartment) and then are resecreted by

retroendocytosis (79). Similarly, the observed transport to the apical compartment seen in the pulse loading experiments could be due to internalization and resecretion of HDL by polarized HKC-8 cells (Fig. 24). Alternately, this may represent a tightly bound, wash resistant HDL subset which dissociates from the cell surface with time.

Transcytosis

Transcytosis is a term and concept coined by Simionescu to define the vesicular transport of macromolecules from one side of a cell to the other, a strategy used by multicellular organisms to selectively move material between two environments without altering the unique compositions of those environments (Fig. 24) (218). It is a basic process shared by most epithelial and endothelial cells. In intestinal cells, transcytosis is a branch of the endocytic pathway, with cargo being internalized via receptor-mediated (i.e., clathrin-coated) mechanisms and progressively sorted away from internalized material destined for other cellular destinations (Fig. 24) (134). The regulation of transtubular transport of proteins from the lumen of the proximal tubule to the peritubular space has received much discussion about the extent and role of either transcellular or paracellular transport (124, 148). Cultured cells exhibit transcellular transport of internalized ligands. Christensen *et al.* suggested that a large fraction of the protein reabsorbed by endocytosis into renal proximal tubule cells was transported intact through the proximal tubule cells by transcytosis (125). An extensive transcytosis from the apical membrane to the basolateral surface takes place in cultured rabbit proximal tubule cells (148) and in MDCK cells (223, 225, 227), constituting about 50% of the protein uptake and without involvement of lysosomes and the Golgi apparatus (124). Electron

microscope level endocytic uptake and intracellular transport in the proximal tubule have been studied for a variety of larger peptides and proteins (130).

In experiments, where ligand was incubated with the cells in the apical media for long time periods, the transport observed could have been due to both paracellular (between the cells) and trancytotic (through the cells) transport (Fig. 24). To measure transport through the cells, we designed pulse-loading experiments where polarized HKC-8 cells were incubated with ligands for one hour. After one hour, the medium was removed from both compartments. Ligand-free medium was added to both compartments and incubated at 37°C for various times. The basolateral transport seen in our pulse loading experiments can be attributed primarily to transcytosis (Fig. 24).

In our pulse-loading experiments, we observed that HDL-protein is secreted more to the basolateral compartment when compared to the apical compartments (Fig. 17). Maximum secretion appears to occur at the 2 hour time point. This is followed by a loss of radioactivity in both the apical and the basolateral compartments with time. Western blot analysis further confirmed that intact apoA-I and whole HDL are secreted to the apical and basolateral sides (Fig. 18). This clearly demonstrates the transcytotic transport of intact apoA-I in human proximal tubule cells (Fig. 24). Similarly, Marino *et al.* showed that passage of RBP (retinol-binding protein) through immortalized rat renal proximal tubule (IRPT) cells occurs by transcytosis after megalin-mediated endocytosis, which provides an alternative pathway for recycling of retinol (229). Similar to our experiments, IRPT cells cultured as polarized monolayers with tight junctions were used on permeable filters in the upper chamber of dual-chambered devices, with megalin expression exclusively on the upper surface. After addition of RBP to the upper chamber

and incubation at 37°C, intact RBP was found in fluids that were collected from the lower chamber. They also found that some RBP was internalized and degraded by IRPT cells, but this process was not appreciably affected by megalin competitors, indicating that RBP endocytosed by megalin was not transported to lysosomes and degraded but rather transcytosed across IRPT cells (229). We saw similar trends with radioactivity and immunochemical tracking, which suggests that intact HDL is transcytosed through HKC-8 cells. Our data is also consistent with other studies which showed significant transcellular transport of ^{125}I-lysozyme and insulin in experiments using isolated and microperfused rabbit proximal tubules (147, 277, 278). Ramalingam and co-workers demonstrated transcytosis and recycling of IgG in polarized MDCK cells (231). Similar to our observations, Kobayashi and co-workers suggested that the pathways for intact human IgG transepithelial transport in polarized human renal proximal tubular epithelial cells (RPTEC) may avoid lysosomal degradation of IgG (230).

To track the lipid constituents in HDL, polarized HKC-8 cells were pulse-loaded with HDL containing radioactive lipids. We observed that [^{3}H]-FC-HDL (Fig. 20) and [^{3}H]-DPPC-HDL (Fig. 22) are secreted to both the apical and basolateral compartments. Both PC and FC are secreted more to the apical side when compared to the basolateral compartment. This suggests that the HDL particles may be retroendocytosed to the apical compartment. However, studies using radioactive lipids and ^{125}I-labeled protein to track the different HDL constituents show a significant transport of HDL-protein and -lipid to the basolateral compartment. Taken together the data may suggest that intact HDL is transcytosed in the PT cells.

All lipoprotein classes exhibit a net negative charge and studies have shown that this charge is due to both the apolipoprotein composition of the lipoprotein and its content of anionic lipids (specifically PI) (261, 279). Previous studies in our laboratory have shown that lipoprotein charge is governed by the conformation of the apolipoprotein and is also directly affected by different charged lipids (260, 261). Incubation of PI with plasma or with ultracentrifugally isolated HDL, LDL, or VLDL *in vitro* has shown that all lipoproteins can spontaneously adsorb PI and become more negatively charged (67). PI is an anionic lipid found in all classes of lipoproteins and accounts for approximately 4% of the total PL in HDL (279).

To study the effect of charge on HDL intracellular metabolism in polarized HKC-8 cells, experiments were undertaken using labeled HDL, HDL+PC or HDL+PI. Electrokinetic analysis of the labeled HDL particles used in the experiment showed that PI and PC enrichment directly affects HDL charge (Fig 23, Panel B). PI renders HDL more negatively charged, while PC makes HDL less negatively charged. Western blots of apical and basolateral media shows that more HDL-PC is transported to the basolateral media when compared to HDL and HDL-PI (Fig. 23, Panel A). PI appears to inhibit secretion of HDL to the basolateral side. An increased negative charge in HDL appears to block transcytosis, while a decreased charge stimulates the transcellular transport of HDL. The data suggest that composition/charge induced changes in lipoprotein transcytosis may be the result of altered interactions between the lipid-enriched lipoproteins and specific cell surface microdomains. HDL charge may directly impact the intracellular cargo sorting machinery which may regulate interfacial interactions with specific receptors on the cell surfaces (244).

Several studies have suggested that electric charge of proteins can be determinant for the cell surface binding (247, 248, 249, 280-283) and that cationic proteins are preferentially taken up (247). Sumpio and Maack proposed the "selective-constraint model" to explain the proximity of the microvilli anionic sites and the accessibility of proteins to these sites, thus facilitating the adsorption of cationic proteins (247). However, even though the luminal membrane of the proximal tubule cells is negatively charged, anionic proteins are also generally reabsorbed very efficiently, as shown for insulin and α2-microglobulin (124). Cell membrane charge may play an important role in the transport of HDL particles to the basolateral compartment, but it is likely that charge-charge interactions with unique cell surface receptors may also affect lipoprotein transport. Experiments in our laboratory have shown that cell surface proteins are affected by lipoprotein charge and that HDL metabolism *in vivo* is directly affected by the electrical charge on HDL particles (284).

PI performs two potentially related functions in HDL particles: that of a surface charge modulator and that of a cell-signaling stimulant (284). Previous experiments suggest that cholesterol metabolic effects of PI are probably mediated through the actions of specific phospholipases, PKC activation, and inositol phosphate-induced fluxes in intracellular calcium levels. Activation of the inositol cascade may result from an increased concentration of intracellular PI and increased substrate for the actions of PI-specific phospholipase C. The inositol signaling path is reported to alter intracellular calcium metabolism through the release of calcium from the endoplasmic reticulum (284). It has also been reported previously that the addition of PI liposomes to the extracellular medium of fetal lung fibroblasts increases calcium uptake into these cells

(285). PI affects lipoprotein metabolism both by controlling interfacial interactions and uniquely regulating intracellular signaling pathways (284). Similarly, HDL charge may effect intracellular signaling pathways and play an important role in vesicle transport. The data suggest that the electrostatic properties of HDL may directly affect the intracellular transport of HDL in PT cells.

Many candidates that regulate transcytosis in polarized epithelial cells are emerging. They are mainly involved in vesicle transport, targeting, and consumption along the transcytotic route (169). Cargo-bearing vesicles involved in transcytosis deliver their contents to the correct target domains using targeting machinery. Morphological, biochemical, and genetic approaches have successfully identified many molecules involved in vesicle targeting, a complex series of molecular events that minimally includes docking and fusion (169). Members of three protein families are central players in vesicle targeting and fusion (286, 287). They are as follows: SNAREs (288, 289), the Sec1/Munc18 proteins and small-molecular-weight GTP-binding proteins (the rabs) (286, 287). Moreover, over a dozen genes have been identified in yeast that are required for TGN to PM transport (290-292), and of these, more than two-thirds of the gene products form a multimeric complex referred to as the exocyst (293). In addition, annexins are suggested to initiate membrane-membrane contact that results in vesicle fusion (294). Dynamins are known to function in the early stages of endocytosis. Interestingly, the prediction is that dynamin is required for the internalization of transcytosing molecules in polarized epithelial cells (169).

In addition to the asymmetric distribution of PM proteins, the polarity of epithelial cells is also reflected in the organization of the cytoskeleton (169). Disruption

of microtubules has been shown to impair the movement of internalized molecules from early endosomes to other compartments. Microtubules are probably not a direct requirement for transcytosis; they likely facilitate delivery by providing the tracks upon which vesicles are translocated (295). The microtubule-based motor molecule, cytoplasmic dynein is also suggested to be a possible regulator of transcytosis (169). The actin cytoskeleton also has a unique organization in many polarized cells (169). In support of the evidence indicating a role for actin in regulating membrane dynamics, recent studies have also implicated specific actin-based motors (myosin motors) as important players in polarized vesicle trafficking. The single-headed, short-tailed myosin I isoform has been localized to the apical brush border of intestinal (296) and kidney cells (297). Myosin VI has also been placed at the apical brush border of the proximal tubule cell line LLC-PK1 and myosin Vb at the apical recycling endosome in MDCK cells. Lipids, like phosphoinositides, PC, cholesterol, and glycosphingolipids have also been shown to be important players in membrane transport (169).

Though many studies have determined possible mechanisms in transcytosis, many things still remain unknown (169). In peripheral endothelial cells, it appears that separate entry points dictate different fates; internalization via coated pits sends cargo to the endocytic pathway whereas caveolae-mediated internalization ensures a transcytotic fate (169). Where and how transcytotic cargo is sorted in these cells remains to be resolved. Intracellular metabolism is an area where further study is needed to make a definitive conclusion.

In summary, the *in vitro* studies with polarized HKC-8 cells provide evidence for the cellular processing of HDL. Our findings show that HDL is readily reabsorbed at the

apical surface of polarized proximal tubule cells and that most of the protein constituents are re-secreted from the opposite, basolateral surface (Fig. 24). Experiments show that there are significant composition-dependent differences in HDL particle processing. HDL apolipoproteins appear to have a stimulatory effect on HDL transport to the basolateral side when compared to HDL-lipids. We observed almost no apolipoprotein degradative products in the apical and basolateral compartments. Interestingly, we observed that ~60% of HDL-protein was transported to the basolateral surface, when compared to ~10% of HDL-lipids. Our data also shows that HDL-charge appears to regulate the salvage of HDL-proteins in the kidney. These studies have shown that more than one pathway may be responsible for HDL trafficking within the PCT cells of the renal cortex. Transcytosis, retroendocytosis and paracellular pathways appear to all be involved in the intracellular metabolism of HDL in renal proximal tubule cells (Fig. 24).

VI. Physiological Relevance

Patients with clinical disorders such as hyperlipidemia, or LCAT deficiency display reduced levels of HDL in their plasma, resulting in a higher risk of atherosclerosis. It is well established that the rate of clearance of HDL from the blood stream is elevated in patients with low HDL levels and that this is associated with changes in the composition and structure of HDL (12, 15, 298, 299). Studies have suggested that HDL particle size is influenced by plasma TG levels and TG lipase activities (300). It has been shown that TG enrichment of HDL may promote lipolysis, which reduces their size (301) and may increase their catabolic rate (12, 104). *In vitro*, the enrichment of HDL with TG and their subsequent hydrolysis with LPL increases their elimination by isolated rabbit kidneys (302). Studies showed that large TG-rich HDL

were cleared markedly slower than small TG-poor HDL in rabbits (14, 303). Saku *et al.* have also shown that large HDL are cleared more slowly than smaller particles in rabbits (304). Increased HDL clearance rates in hypertriglyceridemic subjects actually appear to be associated with the formation of small TG-enriched HDL (305, 306). Previous studies in our laboratory have showed that the inclusion of very large amounts of TG in LpA-I complexes leads to the formation of very small TG-rich Lp2A-I complexes that are depleted in POPC. These particles are more positively charged and are rapidly cleared from rabbit plasma (S. Braschi and D. Sparks, unpublished data). Smaller and more positively charged HDL appears to be cleared from the circulation faster (14).

A number of investigations have shown that electrostatic interactions may be centrally important in the regulation of HDL metabolism. Studies have shown that the remodeling of HDL particles by the enzymes, LCAT, CETP, PLTP, and HL may be affected by HDL surface charge (260, 307-309). Similarly, SR-BI, which binds HDL and is involved in CE selective uptake from HDL, is also electrostatic sensitive and binds anionic PL with a high affinity (310). Studies show that the overexpression of HL in rabbits leads to enhanced HDL clearance (311). HL hydrolyzes HDL-PL, DG, and TG and thereby alters the composition and charge of HDL (308). Conversely, studies show that the overexpression of human LCAT in transgenic rabbits raises the CE content of HDL and decreases apoA-I catabolism from plasma (312). The esterification of FC by LCAT increases HDL particle size (313). LCAT deficiency leads to small HDL and enhanced HDL clearance (314).

Several investigations have shown that the dissociation of apoA-I from HDL leads to the formation of a very small, lipid-poor HDL subclass that has been called

pre-β-HDL (315, 316). Some lines of evidence have suggested that this subclass (pre-β-HDL) may be cleared more rapidly from the plasma by renal filtration (85, 317). This result supports the hypothesis that positively charged, poorly lipidated apoA-I are cleared more rapidly from plasma than a neutral lipid-rich HDL particle (85, 317). Studies in humans concerning the relative rates of clearance of different HDL fractions have been performed. Clearance of large HDL has been reported to be slower (304) than that of small HDL. Therefore, small positively charged HDL appears to be cleared faster in the blood stream.

The organ specific clearance of HDL has been studied by monitoring uptake of labeled apoA-I (16-21, 85, 318, 319) and most studies have shown the liver and kidneys to be the primary sites for apoA-I degradation (16-21, 320). However, the kidney appears to be the most important organ in apoA-I clearance and is more active (per gram tissue) than the liver in the clearance of this protein (16-21, 85). Studies show that the renal proximal tubule of the renal cortex is the major site for the uptake of apoA-I, HDL and most other proteins filtered in the glomeruli (22, 321). A number of investigations conducted in humans and animals show that the kidney glomerular capillary wall exhibits charge selectivity (322, 323). The presence of negatively charged sites within the glomerular capillary wall hinders the filtration of anionic molecules on an electrostatic basis (322, 323). Studies suggest that a decrease in the negative charge on apoA-I will promote renal uptake, and increase apoA-I clearance from plasma. This increased clearance may be related to enhanced renal filtration, poor reabsorption and/or degradation within the tubular apparatus. Our data showing that positively charged HDL is preferentially salvaged appears to contradict *in vivo* studies showing that positively

charged HDL is cleared more rapidly from the circulation. Similarly, studies demonstrate that more positively charged molecules cross the blood brain barrier (BBB) because of the negative surface charge of the luminal endothelial membrane of brain vessels (324). It is well known that the endothelial cell surface bears a negative surface charge at physiological pH. This electrostatic barrier repels negatively charged molecules. It has been known for more than 60 years that the injection of cationic molecules results in an increase in the permeability of cerebral vessels (325). Polycationic protamine sulfate, cationized ferritin, poly-L-lysine, and sulfated dextrans have all been shown to increase the permeability of horseradish peroxidase in rat brain (326-332). In a similar manner, the cationization of proteins, such as albumin, lgG or native histone has resulted in enhanced binding and transport at the blood-nerve barrier (BNB) and the BBB (333-339). Although these studies suggest a mechanism involving absorptive-mediated endocytosis after rapid interaction of the positively charged protein molecule with the negatively charged surface of the endothelial cell, it is not clear if these cationic proteins affect permeability of the BBB or BNB as do the aforementioned cationic molecules. The covalent attachment of naturally occurring polyamines that has been described in a variety of mammalian cells may play a role in allowing charge interactions of the polyamine-modified proteins with the negative surface charge of membranes. The permeability of the BNB and BBB to superoxide dismutase, insulin, albumin, and IgG in normal adult rats was quantified before and after covalent protein modification with the naturally occurring polyamines-putrescine, spermidine, and spermine (324). Positively charged polyamine modification of proteins was shown to dramatically increase the permeability at the BNB and BBB of a variety of proteins. Poduslo and Curran hypothesized that this charge interaction may

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increase the permeability of such cationic proteins by facilitating absorptive endocytosis (324). Surprisingly, they observed that increasing the charge valency on proteins with different naturally occurring polyamines resulted in a significant decrease in the permeability. This finding implies that the observed permeability changes occur by a mechanism other than simple electrostatic interaction involving charge density. They suggest that this increase in transport may be due to the presence of polyamine specific bidirectional carrier mediated transport system (324). Our data showing that positively charged HDL is preferentially absorbed could be due to the proximity of the proximal tubule microvilli anionic sites and the accessibility of proteins to these sites, thus facilitating the adsorption of cationic proteins (247). Lipoprotein charge has also been shown to affect lipoprotein metabolism both by controlling interfacial interactions and uniquely regulating intracellular signaling pathways (284). Similarly, HDL charge may effect intracellular signaling pathways and play an important role in vesicle transport. Christensen *et al.* suggested that a large fraction of proteins reabsorbed by endocytosis into renal proximal tubule cells was transported intact through the proximal tubule cells by transcytosis (130). Since under non-pathological conditions, only small amounts of apoA-I are lost in the urine (101, 321), an active apoA-I/HDL re-absorption process must occur in the kidney proximal tubule to prevent excessive losses of this important molecule.

HDL loss can occur by hepatic clearance and during renal filtration. Studies show that SR-BI is responsible for the selective uptake of CE from HDL (340). CE selective uptake supplies cholesterol to the liver and steroidogenic tissues, for biliary cholesterol secretion and steroid hormone synthesis (340). SR-BI is responsible for the removal of

HDL from the maternal circulation to provide nutrients to the fetus via the placenta, yolk sac, and uterine membrane (341). In addition, current evidence suggests that HDL are the major source for steroid production in adrenal gland, ovary and testicular Leydig cells (341).

Our data suggests that the apical to basolateral transport could serve to deliver intact HDL/apoA-I to the tubular basement membrane, and consequently back to the circulation in accordance with the proposed role of the kidney in HDL metabolism (Fig. 24). The identification and isolation of regulators of intracellular traffic that could enhance basolateral delivery and secretion of HDL in renal tubule cells may have some therapeutic value in HDL-deficient states, as their administration could serve to prolong HDL half-life in the blood plasma.

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Awards and Memberships

- Entrance Award, University of Ottawa, Department of Biochemistry, Microbiology and Immunology, 2001-2002 and 2002-2003
- M.Sc. Admission Scholarship, University of Ottawa, Department of Biochemistry, Microbiology and Immunology, BMI, 2001-2002
- Member of the Heart Institute Graduate Students Association (HIGSA), 2001-present
- Thesis nominated for "Young Scientist's Awards-Community Nutrition" by Nutrition Society of India, India (1992-1993).
  Thesis title: Development and Evaluation of Soya Based Weaning Food Formulations (1992)

Presentations (oral and poster)

- M.Sc. Graduate Students Research Presentation, University of Ottawa (2003)
- Research presentation, Lipoprotein Group, University of Ottawa Heart Institute (2003)
- Poster day, Department of Biochemistry, Microbiology and Immunology (2002)
- Canadian Lipoprotein Conference, Banff, Alberta (2002)

Publications

Breznan, D., **Veereswaran, V.**, Viau, F.J., Neville, T.A., and Sparks, D.L. 2004. The lipid composition of HDL affects its reabsorption in the kidney by proximal tubule epithelial cells. *Biochem. J.* (In press).

Contribution of Collaborators

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