MOLECULAR BASIS OF HDL-MEDIATED ENDOTHELIAL CELL MIGRATION AND REENDOTHELIALIZATION

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To my family for their constant love and encouragement

MOLECULAR BASIS OF HDL-MEDIATED ENDOTHELIAL CELL MIGRATION AND REENDOTHELIALIZATION

by

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Publication No. _____

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Vascular disease risk is inversely related to circulating levels of high density lipoprotein (HDL) cholesterol. The atheroprotective nature of HDL is attributed mainly to its role in reverse cholesterol transport (RCT). However, recent reports of human and animal studies have suggested that the atheroprotective nature of HDL is not sufficiently explained by RCT. Therefore, the mechanisms by which HDL provides vascular protection are unclear. The disruption of endothelial monolayer integrity is an important contributing factor in

multiple vascular disorders, and vascular lesion severity is tempered by enhanced endothelial repair. In these studies we show that HDL stimulates endothelial cell migration *in vitro* in a nitric oxide-independent manner via scavenger receptor B type I (SR-BI)-mediated activation of Rac GTPase. This process does not require HDL cargo molecules, and it is dependent on the activation of Src kinases, phosphatidylinositol 3-kinase, and p44/42 mitogen-activated protein kinases. Rapid initial stimulation of lamellipodia formation by HDL via SR-BI, Src kinases and Rac is also demonstrable. Paralleling the *in vitro* findings, carotid artery reendothelialization following perivascular electric injury is blunted in apolipoprotein A-I null (apoA-$I^{-/-}$) mice, and reconstitution of apoA-I expression rescues normal reendothelialization. Furthermore, reendothelialization is impaired in SR-$BI^{-/-}$ mice. Thus, HDL stimulates endothelial cell migration via SR-BI-initiated activation of Rac GTPase, and HDL and SR-BI promote reendothelialization *in vivo*, revealing that signaling by the HDL-SR-BI tandem has a potent beneficial impact on the cardiovascular system.

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Seetharam, D., Mineo, C., Gormley, A.K., Gibson L.L., Vongpatanasin, W., Chambliss, K.L., Hahner, L.D., Cummings, M.L., Kitchens, R.L., Marcel, Y.L., Rader D.J., Shaul, P.W., 2005. HDL Promotes Endothelial Cell Migration and Reendothelialization via Scavenger Receptor-B Type I. *Circ. Res.* In Press.

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LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette A1
aa	amino acid
BAEC	bovine aortic endothelial cells
BP	blood pressure
CETP	cholesteryl ester transfer protein
CLAMP	C-terminal linking and modulating protein
CRP	C-reactive protein
cGMP	cyclic guanine monophosphate
DMEM	Dulbecco's modified Eagle's medium
eNOS	endothelial nitric oxide synthase
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GST	glutathione-S-transferase
HDL	high density lipoprotein
HU	hydroxyurea
LCAT	lecithin:cholesterol acetyltransferase
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
L-NAME	Nω-Nitro-$_L$-arginine methyl ester

LPDS	lipoprotein deficient serum
MAPK	p44/42 mitogen activated protein kinase
MFLM91U	mouse endothelial cells from embryonic lung mesenchyme
MLC	myosin light chain
MLCK	myosin light chain kinase
NO	nitric oxide
PAK	p21-activated kinase
PI3K	phosphatidylinositol 3-kinase
PDZK1	PDZ domain-containing protein
POPC	1-palmitoyl-2-oleoylphosphatidylcholine
RCT	reverse cholesterol transport
RNA	ribonucleic acid
S-1-P	sphingosine-1-phosphate
SR-BI	scavenger receptor B type I
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein

CHAPTER ONE: Introduction

High Density Lipoprotein and Atheroprotection

The risk of cardiovascular disease is inversely related to circulating levels of high density lipoprotein (HDL) cholesterol (Gordon and Rifkind, 1989). The Framingham Heart Study demonstrated that HDL is a potent predictor of coronary artery disease risk in men and women independent of low density lipoprotein (LDL) cholesterol levels (Franceschini, 2001; Gordon et al., 1977). In addition to being associated with reduced coronary artery disease, greater apoA-I and HDL levels correlate with decreased neointima formation and restenosis after coronary angioplasty (Dzavik et al., 1995; Reis et al., 1991). HDL levels are not only an important marker for cardiovascular risk, increased HDL levels reduce cardiovascular mortality and morbidity (Fidge, 1999; Gordon and Rifkind, 1989).

Until recently, the atheroprotective nature of HDL was mainly attributed to its classical role in reverse cholesterol transport (RCT). RCT is the delivery of cholesteryl esters by HDL from peripheral tissues to the liver and steroidogenic organs. A particular subfraction of HDL has the special ability to remove cholesteryl esters from peripheral cells and transport the accumulated cholesteryl esters to the liver by binding to the high-affinity HDL receptor scavenger receptor B type I (SR-BI) on the hepatocyte cell surface (Tall, 1998). Although RCT has

been a widely accepted method of HDL action, recent reports of human and animal studies have suggested that the atheroprotective nature of HDL is not sufficiently explained by RCT. As shown by Groen et al., ATP binding cassette A1 (ABCA1) null mice which have decreased HDL levels do not have decreased delivery of cholesterol from the plasma to the liver (Groen et al., 2001). Additional studies have indicated that in mouse models with decreased HDL levels centripetal cholesterol flux is not altered (Jolley et al., 1998; Osono et al., 1996). As such, the basis for HDL-mediated atheroprotection remains poorly understood.

Metabolism of the HDL Particle

HDLs are a fraction of serum lipoproteins and have a density range of 1.063-1.21 g/ml. The majority of circulating HDL particles is spherical with a diameter of 5-17 nm. They consist of apolipoproteins, cholesterol, phospholipids and triglycerides (**Figure 1-1**). Apolipoproteins and phospholipids are found on the outer shell of the particle and triglycerides and esterified cholesterol are found in the core. Apolipoprotein A-I (apoA-I) is the major apolipoprotein associated with HDL.

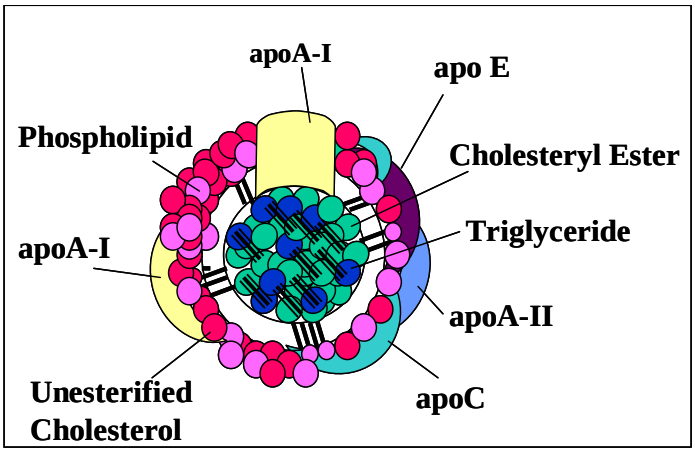

Figure 1-1. The HDL particle. The HDL particle consists of apolipoproteins, cholesterol, phospholipids and triglycerides. ApoA-I is the major apolipoprotein associated with HDL.

The HDL fraction in human plasma is heterogeneous consisting of lipid-poor and lipid-rich HDL particles. Prebeta-migrating lipid-poor apoA-I is synthesized in the liver or intestine or it may be released from spherical HDL during the process of remodeling. Phospholipids are picked up by prebeta-migrating, lipid-poor apoA-I in the extracellular space by binding to ABCA1 leading to the formation of discoidal apoA-I-containing HDL (**Figure 1-2A**). The resulting complex is an efficient acceptor of unesterified cholesterol from cell membranes. The unesterified cholesterol in HDL is esterified by lecithin:cholesterol acyltransferase (LCAT) which transfers an acyl group from phosphatidylcholine to cholesterol (**Figure 1-2B**). LCAT also promotes the fusion of small, spherical HDL particles to form larger particles which contain

apoA-I (**Figure 1-2C**). These larger alpha-migrating (spherical) particles represent the main subpopulations of HDL that circulate in the normal human plasma (Barter, 2002). Other enzymes such as cholesteryl ester transfer protein (CETP) transfer cholesteryl esters from HDL to other lipoproteins such as LDL, and triglycerides from LDL to HDL. Fusion of HDL particles can result in the release of prebeta-migrating lipid-poor apoA-I that can receive phospholipids via ABCA1 and complete the cycle (**Figure 1-2D**). They can also be incorporated into pre-existing HDL particles or be excreted by the kidney (Ballantyne and Nambi, 2004; Barter, 2002; von Eckardstein et al., 2000).

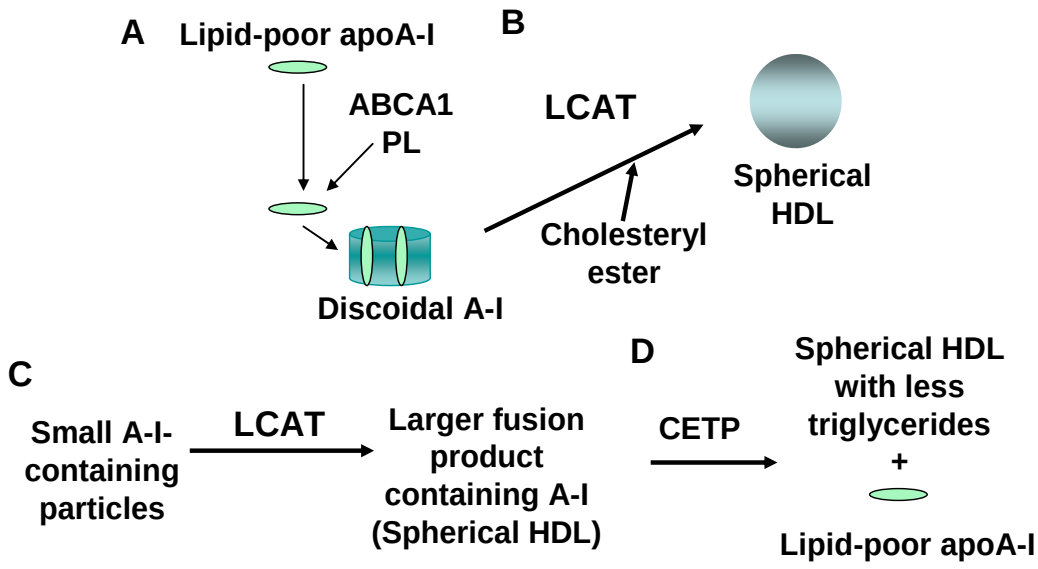

Figure 1-2. Metabolism of HDL.

ApoA-I

The major HDL apolipoprotein apoA-I is essential for the cholesterol uptake properties of HDL. ApoA-I is synthesized in the liver and intestine and is the most abundant protein component of HDL. The absence of apoA-I results in reduced cholesteryl ester transfer from cells to HDL but does not affect the binding affinity of HDL to its high affinity receptor SR-BI (Temel et al., 2002). The correlation between HDL levels and decreased risk of atherosclerosis has promoted several groups to study the role of apoA-I in atherogenesis. Expression of a human apoA-I transgene in C57BL/6 mice leads to increased plasma apoA-I and HDL levels. These mice are protected from diet-induced development of fatty streak lesions compared to C57BL/6 mice without the transgene (Rubin et al., 1991). Similarly, expression of a human apoA-I transgene in mice lacking apoE leads to decreased atherosclerotic lesions on a chow diet (Paszty et al., 1994; Plump et al., 1994). Cholesterol-fed rabbits develop spontaneous atherosclerosis within 12 weeks of feeding. HDL-cholesterol isolated from healthy rabbits or purified apoA-I injected into cholesterol-fed rabbits decrease aortic fatty streak formation (Badimon et al., 1990; Miyazaki et al., 1995). Additional studies have used apoA-I-containing adenovirus to increase the plasma concentration of apoA-I and HDL in mice. Liver-directed gene transfer of an apoA-I-containing adenovirus into mice of atherogenic backgrounds such as $apoE^{-/-}$ or $LDLR^{-/-}$ mice leads to decreased neointima formation after wire injury

and decreased atherosclerotic lesions in the aortic arch and aortic root (De Geest et al., 1997; Tangirala et al., 1999). To test the role of apoA-I in remodeling of preexisting lesions, aortic grafts with advanced lesions from apoE$^{-/-}$ mice were implanted into apoE$^{-/-}$ mice with or without an apoA-I transgene. The progression of atherosclerosis in the lesions was prevented in the mice with increased expression of apoA-I (Rong et al., 2001). Collectively, these studies indicate that increased apoA-I and HDL expression in the plasma leads to decreased atherogenesis and prevention of lesion progression in pre-existing lesions.

ApoA-I$^{-/-}$ mice have one third of the level of HDL-cholesterol and are grossly deficient in alpha-migrating HDL particles compared to wild type mice (Williamson et al., 1992). The importance of apoA-I is exemplified in apoB transgenic/apoA-I$^{-/-}$ mice which have increased lesions on an atherogenic diet compared to apoB transgenic mice alone (Hughes et al., 1997; Voyiaziakis et al., 1998). In addition to these studies, apoA-I$^{-/-}$/LDLR$^{-/-}$ mice on a chow diet develop significantly greater oxidant stress and atherosclerosis compared to LDLR$^{-/-}$ mice alone (Moore et al., 2003). Collectively, the results from these studies clearly display an atheroprotective role of apoA-I.

SR-BI

The high affinity HDL receptor SR-BI belongs to the class B scavenger receptor family of proteins. It is detected as an 82 kD protein by gel

electrophoresis although the amino acid (aa) sequence encodes a 509 aa protein mainly due to extensive *N*-glycosylation (Babitt et al., 1997). The protein sequence contains a putative membrane-spanning region at each end of the protein and a large extracellular domain with multiple *N*-glycosylation sites (**Figure 1-3**) (Krieger, 2001).

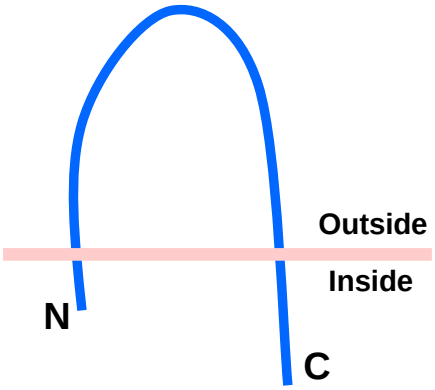

Figure 1-3: Model of the topology of SR-BI.

Several groups have detected SR-BI enrichment in cholesterol-rich membrane microdomains called caveolae of various cell types (Babitt et al., 1997; Graf et al., 1999; Uittenbogaard et al., 2000; Yuhanna et al., 2001). SR-BI is highly expressed in the liver, adrenal gland, ovary, and testis, tissues highly dependent on cholesterol and cholesteryl esters for bile acid synthesis and steroidogenesis. SR-BI is also expressed in other tissues and cell types including the brain, intestine, placenta, macrophages, keratinocytes, adipocytes, platelets, smooth muscle cells, and endothelial cells (Connelly and Williams, 2004).

Acton et al. isolated the cDNA for SR-BI by expression cloning and found that SR-BI transfected into LDL receptor-negative cells binds to native and modified LDL. In addition to these lipoproteins, it binds with high affinity to HDL and mediates the uptake of cholesteryl esters from HDL into the cells. HDL competes effectively with LDL for the binding to SR-BI, whereas LDL only partially competes with HDL for binding to SR-BI. Unlike HDL labeled with [^{3}H] cholesteryl ether, ^{125}I-labeled HDL protein is not taken up by SR-BI into the cells (Acton et al., 1996) indicating that SR-BI mediates selective transfer of cholesterol by a mechanism distinct from the LDL receptor. Additional studies have revealed that HDL binds to the extracellular domain of SR-BI in hepatocytes which facilitates transfer of lipid from HDL to the plasma membrane. High affinity binding of HDL is not sufficient for lipid uptake by SR-BI. Efficient lipid uptake by SR-BI requires proper orientation of apoA-I and the extracellular domain of SR-BI (Connelly and Williams, 2004).

The role of SR-BI in cell culture studies in inducing selective cholesterol uptake from HDL led to the hypothesis that SR-BI may play a role in mediating the anti-atherogenic effects of HDL. This hypothesis was tested in several studies involving the genetic manipulation of SR-BI in mice. Rigotti et al. were the first to generate mice with a targeted null mutation in the SR-BI gene (Rigotti et al., 1997). SR-BI$^{-/-}$ mice have increased plasma total cholesterol mainly due to an increase in cholesterol in the HDL fraction (**Figure 1-4**). In contrast to the

increased plasma cholesterol, the plasma apoA-I content is similar in the mutants compared to the wild type mice. Immunoblot analysis of the different fractions in the HDL peak show increased apoE and decreased apoA-II levels in the SR-BI$^{-/-}$ HDL. Lipoprotein profiles show broader peaks of HDL in SR-BI$^{-/-}$ mice compared to SR-BI$^{+/-}$ or SR-BI$^{+/+}$ mice indicative of larger and more heterogeneous HDL particles.

Further analysis of the SR-BI$^{-/-}$ mice demonstrated SR-BI requirement for reproduction in female mice. Although SR-BI$^{-/-}$ female mice produce oocytes, they are infertile due to diminished viability of the oocytes and embryos (Trigatti et al., 1999). Reduction in HDL cholesterol content by treating SR-BI$^{-/-}$ mice with probucol or crossing them with apoA-I$^{-/-}$ mice results in increased fertility and pup yield (Miettinen et al., 2001). These results indicate that abnormal lipoprotein metabolism is responsible for infertility in SR-BI$^{-/-}$ females which can be restored with appropriate modification of the structure, composition and abundance of the lipoproteins.

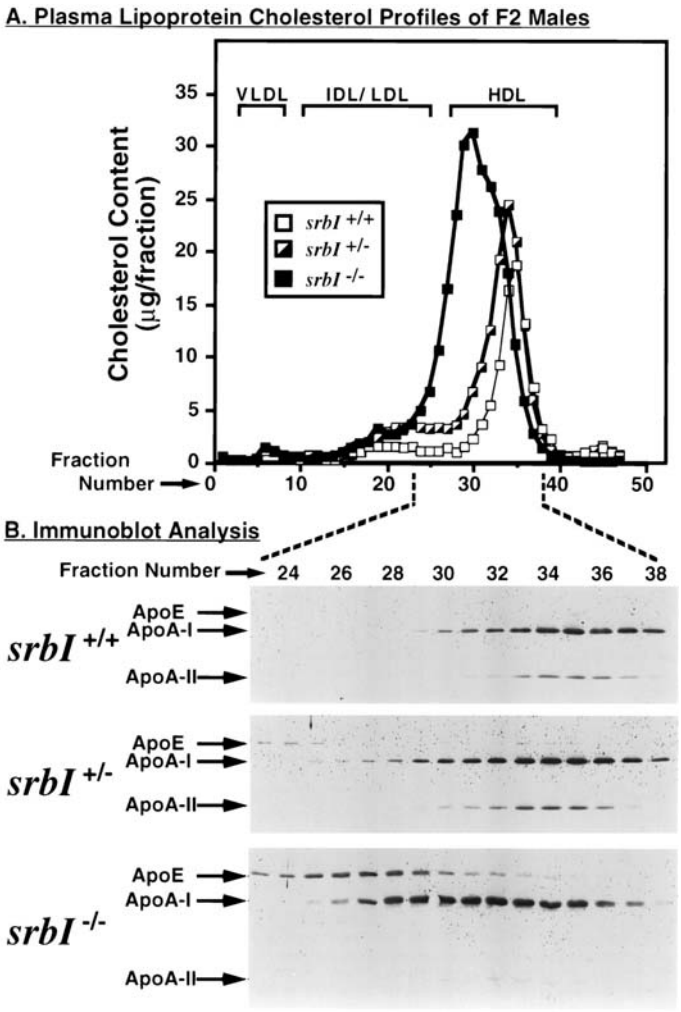

Figure 1-4: Analysis of lipoproteins from SR-BI$^{+/+}$, SR-BI$^{+/-}$, and SR-BI$^{-/-}$ mice. FPLC (A) and immunoblot analysis (B) of lipoproteins in SR-BI$^{+/+}$, SR-BI$^{+/-}$, and SR-BI$^{-/-}$ mice. Figure adapted from Rigotti et al., 1997. Copyright ® 1997 by National Academy of Sciences.

In addition to mediating fertility in female mice, SR-BI is required for preventing myocardial infarction and cardiac dysfunction. SR-BI deficiency in mice leads to increased lipid deposition in the aorta after a high fat diet compared to SR-BI wild type mice (Van Eck et al., 2003). SR-BI$^{-/-}$ mice crossed with apoE^{-}

$^{/-}$ mice have increased plasma cholesterol, larger HDL-cholesterol and an accelerated onset of atherosclerosis compared to SR-BI$^{-/-}$ or apoE$^{-/-}$ mice. At 4-7 weeks of age, no atherosclerotic lesions are present in apoE$^{-/-}$ mice. However, SR-BI$^{-/-}$/apoE$^{-/-}$ mice contain lesions in the aortic root, aortic sinus and coronary arteries (Trigatti et al., 1999). As observed by Braun et al., loss of SR-BI in apoE$^{-/-}$ mice also leads to a striking increase in lipid-rich coronary artery occlusions and atherosclerotic lesions in the aortic sinus as early as 4 weeks after birth. In addition to these abnormalities, the mice develop extensive areas of myocardial infarction and fibrosis, and increased heart weight leading to premature death of all mice by 8 weeks of age (Braun et al., 2002). In addition to deletion of SR-BI in mice, the role of SR-BI in atherosclerosis has also been tested in mice susceptible to atherosclerosis by overexpressing SR-BI using a transgene or liver-directed gene transfer of an SR-BI-containing adenovirus. Low-level expression of the SR-BI transgene in apoB transgenic mice on an atherogenic diet results in an ~2-fold decrease in the development of fatty streak lesions compared to apoB transgenic mice without the SR-BI transgene (Ueda et al., 2000). LDLR$^{-/-}$ mice given a western diet develop atherosclerotic lesions within 6 weeks of start of the diet. Hepatic overexpression of SR-BI in LDLR$^{-/-}$ mice using an SR-BI-containing adenovirus results in significantly smaller lesions compared to mice given empty virus (Kozarsky et al., 2000). Collectively, these studies indicate

that the presence of SR-BI leads to decreased atherogenesis, but the mechanisms behind the atheroprotective nature of SR-BI remain poorly understood.

HDL and signaling to eNOS

A key early event in the pathogenesis of atherosclerosis is the diminished availability of the vasodilatory molecule nitric oxide (NO). NO is produced by endothelial nitric oxide synthase (eNOS) in endothelial cells lining the vasculature. A downregulation of eNOS activity is correlated with endothelial dysfunction resulting in decreased vasodilation, increased leukocyte adhesion, smooth muscle cell proliferation and vascular oxidative stress (Gewaltig and Kojda, 2002; Shaul, 2003). eNOS, which is localized to the caveolae membrane in endothelial cells, generates NO in response to various stimuli by conversion of L-arginine to L-citrulline and NO. NO activates soluble guanylate cyclase to produce the second messenger cyclic guanosine monophosphate (cGMP). cGMP-dependent activation of protein kinases and the subsequent reduction in intracellular calcium concentration results in vasodilation.

In addition to the classical properties of HDL and SR-BI in reverse cholesterol transport, this ligand-receptor pair has been shown to induce the production of NO. HDL activates eNOS in endothelial cell caveolae in an SR-BI-dependent manner (Yuhanna et al., 2001). As shown by Mineo et al., this activation requires the activities of Src kinases, and the sequential activation of

phosphatidylinositol 3-kinase (PI3K) which in turn activates Akt kinase and p42/44 mitogen-activated protein kinase (MAPK) (**Figure 1-5**). Akt kinase phosphorylates eNOS on serine 1179 which allows for eNOS activation. Although MAPK is not required for eNOS phosphorylation, it is necessary for the activation of eNOS by an unknown mechanism (Mineo et al., 2003).

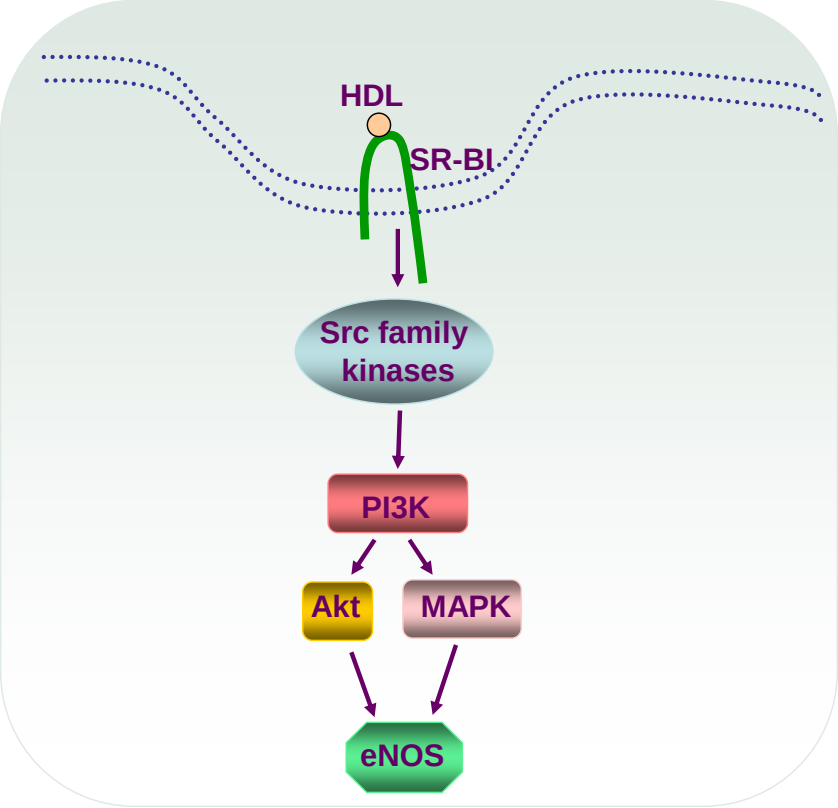

Figure 1-5: HDL activation of eNOS. HDL activation of eNOS requires the activities of Src family kinases, PI3K/Akt and MAPK.

The proximal events leading to HDL activation of the kinases upstream of Src family kinases are unknown. Although it is clear that SR-BI is required for

the action of HDL, the binding partners/signaling complexes associated with SR-BI necessary for HDL signaling are elusive.

PDZK1

Recently, candidates such as PDZK1 (PDZ domain-containing protein), also known as CLAMP (C-terminal linking and modulating protein), have come into consideration as SR-BI binding partners. PDZK1 is a 70 kD protein which contains four PDZ domains. Early studies identified PDZK1 as a protein in rat liver extracts which interacts with the last 15 aa in the cytoplasmic C-terminus of SR-BI (Ikemoto et al., 2000). Further studies identified an SR-BI mutant missing the last aa (SR-BIdel509) unable to interact with PDZK1. Expression of wild type SR-BI in the liver of mice results in >20-fold increase in the protein and decreased plasma cholesterol whereas expression of the SR-BIdel509 transgene in the liver results in moderate expression of the protein and no change in the plasma cholesterol mainly due to decreased expression of SR-BIdel509 on the cell surface (Silver, 2002). Therefore, PDZK1 is required for the translocation of SR-BI to the plasma membrane. Since PDZK1 is required for efficient expression of SR-BI on the cell surface and since PDZ-domain-containing proteins act as protein interaction modules bringing together large protein complexes (Nourry et al., 2003; Sheng and Sala, 2001), it is possible that PDZK1 is involved in HDL-mediated signaling to eNOS via SR-BI. This hypothesis was recently tested in a

report by Assanasen/Mineo et al. in which we found that HDL-induced stimulation of eNOS requires cholesterol efflux to HDL, the C-terminal transmembrane domain of SR-BI which directly binds cell cholesterol, and the C-terminal PDZ-interacting domain of SR-BI (Assanasen et al., 2005). SR-BI mutants or chimeras which lack the PDZ-interacting domain of SR-BI or contain an altered C-terminal transmembrane domain do not transduce the signal to eNOS. However, SR-BI chimeras with a CD36 extracellular domain but intact C-terminal transmembrane and PDZ-interacting domains of SR-BI are capable of transducing the signal to eNOS. Even though SR-BI extracellular domain is required for SR-BI-mediated cholesteryl ester efflux, the observations suggest that the level of efflux mediated by CD36 is sufficient to mediate HDL signaling if the appropriate SR-BI domains are present to transduce signal. These results suggest the involvement of PDZK1, clarifying the mechanism by which the HDL-SR-BI pair signals to eNOS.

Endothelial Injury

In addition to a diminished availability of NO in the vascular wall, the endothelial layer is subjected to numerous stresses such as exposure to cytokines, shear stress, reactive oxygen species and mechanical stresses. Endothelial cells in lesion-prone areas are characterized by enhanced cell turnover rates. Atherosclerosis preferentially develops in these areas suggesting a mechanistic

link between increased cell death and plaque development (Rossig et al., 2001). Disruptions of endothelial cell monolayer integrity, either by gross denudation related to a vascular intervention or gap formation between cells, place the arterial wall at greater risk for vascular disease (Cunningham and Gotlieb, 2005; Gotlieb and Silver, 2001; Ross, 1993). In addition, whereas repeated endothelial removal worsens the severity of vascular lesions (Niimi et al., 1994) enhanced reendothelialization blunts lesion formation (Rossig et al., 2001; Shizukuda et al., 1999; Tournier et al., 1984; Werner et al., 2003).

Endothelial Cell Migration

In the event of the loss of endothelial monolayer integrity, the cytoskeletal network is activated to regulate cell spreading and migration into the wound. In the initial stages, endothelial cells at the wound edge undergo partial detachment from their neighbors and spread by lamellipodia extension into the wound. The cells then enter the second stage in which microfilaments are organized in a parallel orientation relative to the wound edge which allows the cells to spread laterally. The last stage, which is the initiation of cell migration, is characterized by organization of the microfilaments perpendicular to the wound edge (Lee and Gotlieb, 2003). Cell migration can be divided into four separate steps: lamellipodium extension, formation of new adhesions, cell body contraction, and tail retraction (**Figure 1-6**).

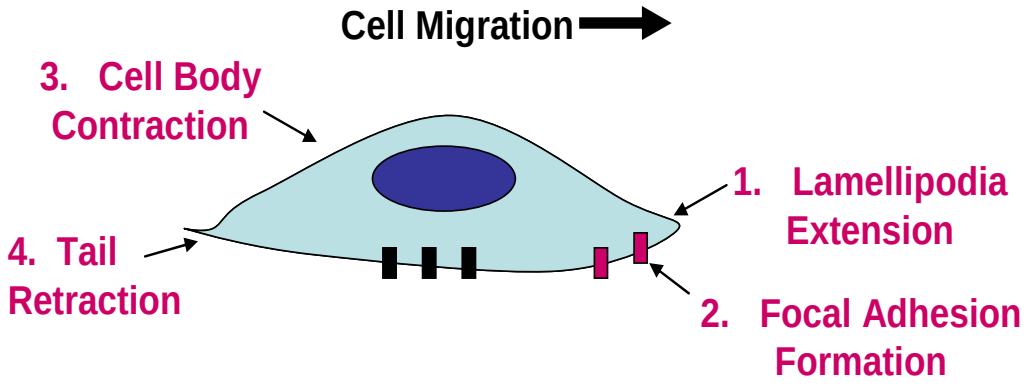

Figure 1-6. Model for the steps of cell migration.

Among other proteins, the Rho GTPases (Rho, Rac and Cdc42), which regulate specific cytoskeletal structures (Hall, 1998), play key roles in the regulation of each step in cell migration (Nobes and Hall, 1995). Rho, Rac and Cdc42 are GTPases which are activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase-activating proteins (GAPs) and regulate the formation of stress fibers, lamellipodia, and filopodia, respectively (**Figure 1-7**).

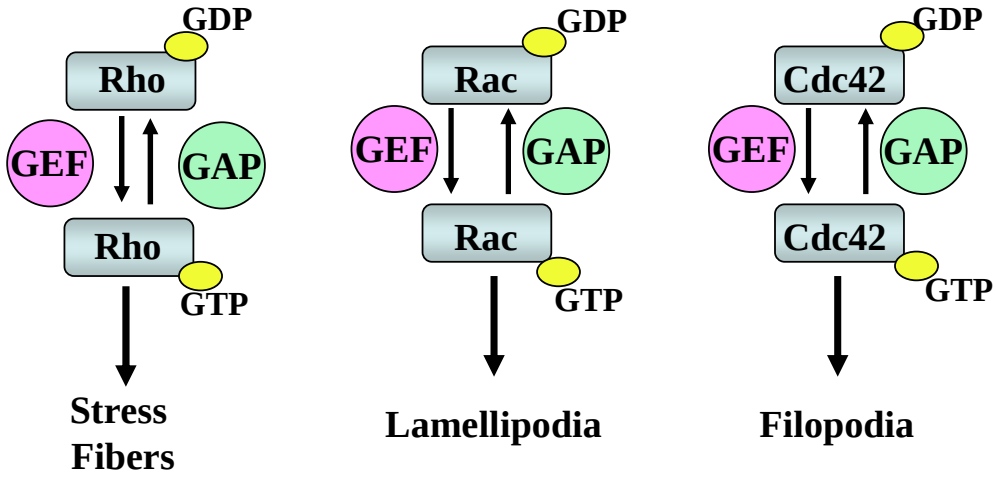

Figure 1-7: GTPase cascades involved in cytoskeletal reorganization.

The initiation of cell movement involves extension of lamellipodia which requires actin polymerization. Rac is required for lamellipodia formation induced by growth factors, cytokines and extracellular matrix proteins (Figure 1-6). Inhibition of Rac activity or overexpression of constitutively active Rac results in attenuation of cell migration (Allen et al., 1998; Nobes and Hall, 1999). Rac activation is often dependent on the activity of PI3K. The products of PI3K, PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 are enriched at the leading edge of migrating cells, a region where Rac is preferentially localized (Kraynov et al., 2000; Servant et al., 2000). Activated Rac stimulates lamellipodium extension by regulating several targets. Rac stimulates actin polymerization by activating the Arp2/3 complex which initiates branching of actin filaments (Miki et al., 2000). In addition to activating the Arp2/3 complex, Rac can stimulate actin polymerization by

triggering the uncapping of actin filaments at the plasma membrane. Capping proteins are removed from the barbed ends of actin filaments by PtdIns(4,5)P_2 resulting in increased actin polymerization (Tolias et al., 2000). Rac can also affect the rate of actin depolymerization by stimulating the activity of LIM-kinase via the Rac/Cdc42 target p21-activated kinase (PAK), which is essential for cell motility (Edwards et al., 1999; Ridley, 2001). PAK has other targets in the cell such as myosin light chain kinase (MLCK). It phosphorylates and inactivates MLCK leading to decreased MLC phosphorylation which results in regulation of cell contraction.

Focal complexes mediate the attachment of the extending lamellipodium to the extracellular matrix. There is continuous cross-talk between the integrins at the leading edge and Rac GTPase which keeps both sets of proteins active. Focal complexes/adhesions are disassembled, which is triggered by activated PAK, as the cell body moves over them. Rho acts via Rho kinases to phosphorylate MLC resulting in cell body contraction. The last step which can often be the rate-limiting step in migration is tail detachment. Focal adhesions at the rear of the cell are disassembled which allows for detachment of the tail and movement of the cell, thus completing the cycle (Figure 1-6).

Since HDL stimulates signaling pathways which include Src kinases, PI3K/Akt, MAPK and eNOS, proteins known to be involved in cell migration, their involvement in HDL-induced endothelial cell motility is possible. In the

following set of studies, we determine the role of HDL in endothelial cell migration and elucidate the mechanisms by which HDL induces migration in primary bovine aortic endothelial cells. The proteins required for HDL-mediated migration and their placement in the signaling cascade initiated by HDL is determined. In addition to the *in vitro* studies, the physiological relevance of the results is evaluated *in vivo* using apoA-I$^{-/-}$ and SR-BI$^{-/-}$ mice.

CHAPTER TWO: Role of HDL in endothelial cell migration, lamellipodia formation, and Rac activation

Introduction

A key initiating event in atherogenesis is the disruption of endothelial cell integrity. This can occur due to endothelial exposure to cytokines, disturbed shear stress, decrease in NO production or mechanical denudation. These factors lead to a dysfunctional endothelial barrier allowing infiltration by macrophages leading to plaque development, plaque rupture and eventual thrombosis (Rossig et al., 2001) (**Figure 2-1**).

These events leading to arterial occlusion may be prevented by efficient repair of the endothelium after injury. Endothelial repair initially involves the migration of endothelial cells from the wound edges followed by regeneration of the cells (Lee and Gotlieb, 2003). In order to further understand the basis of HDL-mediated atheroprotection, we determined if HDL induces endothelial cell migration after wounding *in vitro*. We used the scratch wound assay which is an established method for emulating wounding of cells in culture. In addition to the 24-hour migration readout, we determined if HDL induces rapid cytoskeletal rearrangements which are necessary for cell movement.

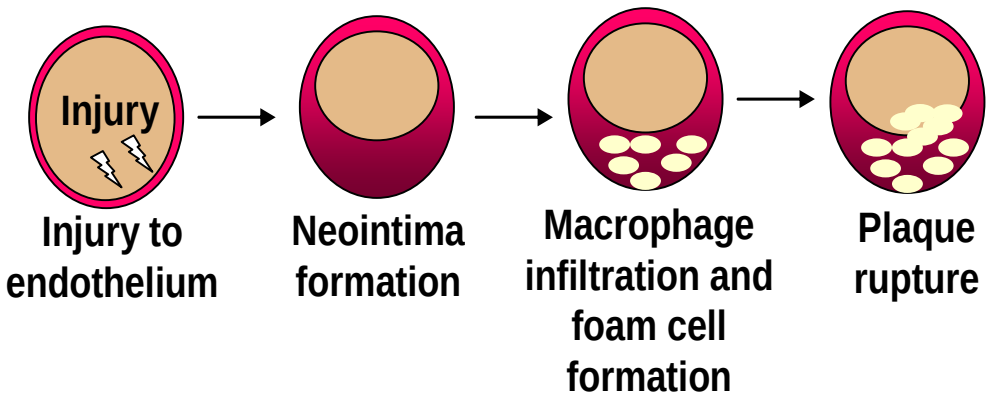

Figure 2-1: Events in the vascular wall after endothelial injury

Methods

Cell Culture: Bovine aortic endothelial cells (BAEC) were harvested from fresh aortas. The aortas were transported from a local slaughterhouse in cold RPMI 1640 with antibiotics and antimycotics. After extensive washing of the outside and inside of the aortas with cold phosphate-buffered saline, the lumen of the aortas were incubated in 2.5 mg/ml collagenase in RPMI 1640 at room temperature for 8 min. Cells were collected and plated in gelatin-coated flasks in EGM-2 medium (Cambrex Bioscience) with 5% fetal bovine serum (FBS) (Sigma). The cells maintained a cobble-stone-like morphology and demonstrated uptake of DiL-labeled acetylated low density lipoprotein (DiL-ac-LDL) through several passgages. BAEC were cultured in EGM-2 medium with 5% FBS, and used between passages 5 and 9.

Endothelial Cell Migration Assay: BAEC were grown to near-confluence in 60-mm dishes and placed in 1% lipoprotein deficient serum (LPDS, provided by Drs. J. Goldstein and M. Brown, UT Southwestern) in Dulbecco's modified Eagle's medium (DMEM) (Sigma) for 16 h, and a defined region of cells was removed with a single-edged razor blade. Cells were treated with HDL (20-100 μg/ml) in DMEM + 1% LPDS and 24 h later fixed with 3% paraformaldehyde (Sigma), permeabilized in 0.2% Triton X-100 (Bio-Rad Laboratories), stained with

hematoxylin (Fisher Scientific), and viewed under an inverted microscope (Zeiss Axiovert 100M). The number of cells which had migrated past the wound edge was quantified in a minimum of 3 high power (100X) fields. In selected studies, cells were treated with 50 ng/ml vascular endothelial growth factor (VEGF) (Calbiochem), 50 μg/ml Lp2A-1 particles, or 1 μM S-1-P. In other studies, cells were treated with 1 μM S-1-P or 50 μg/ml HDL in the absence or presence of 50 ng/ml Ptx and migration was evaluated. Results were confirmed in a minimum of three independent experiments.

Preparation of Native and Reconstituted HDL Particles: Human HDL was provided by Drs. J. Goldstein and M. Brown, UT Southwestern (Stangl et al., 1999). Lp2A-I were prepared from POPC, cholesterol, and recombinant apoA-I at molar ratios of 40:0:1, 80:0:1, or 80:5:1 as reported previously (Sparks et al., 1992) (Provided by Dr. Y. Marcel, University of Ottawa). Additions of native HDL and reconstituted HDL particles in the migration assays were based on their protein content.

Cytoskeletal Changes in Endothelial Cells: BAEC were plated on glass coverslips (Fisher Scientific), grown to ~70% confluence and placed in DMEM for 16 h. Cells were treated with HDL (50 μg/ml; 1 to 30 minutes), fixed in 3% paraformaldehyde, permeabilized in 0.2% Triton X-100, stained with Alexa 568-

phalloidin (Molecular Probes, Inc.), and viewed under a fluorescent microscope at 630X magnification. The percent of cells with lamellipodia was quantified in a minimum of 100 total cells. For statistical analyses, values from a minimum of three independent experiments were combined.

Rac Activity Assay: BAEC were placed in DMEM for 16h and treated with 50 μg/ml HDL for 1-30 min. After treatment, Rac activity was measured as previously described (Eriksson et al., 2003). Briefly, after treatment, cells were harvested in cold 1X lysis buffer [Tris HCL (pH 7.5): 50 mM; glycerol: 10%; $MgCl_2$: 10 mM; NaCl: 150 mM; Triton X-100: 1%]. The nuclear pellet was centrifuged at 13,000 rpm for 10 min at 4º C. The supernatants were incubated with the fragment of p21-activated kinase (PAK) containing the Rac-binding domain conjugated to glutathione-S-transferase (GST-PAK) (provided by Dr. F. Grinnell, UT Southwestern). The complex was collected by centrifugation after 3 washes in 1X lysis buffer and resuspended in 1X SDS sample buffer. Active and total Rac were detected by immunoblot analysis with anti-Rac antibody (Upstate Biotechnology). Results were confirmed in four independent experiments.

siRNA Preparation and Transfection: A double-stranded (ds) RNA with sequence 5'-UGCGUUUCCUGGAGAAUAU-3' was designed to target the open reading frame of bovine Rac1 (Gonzalez et al., 2004). A dsRNA with sequence

5’-AGUUAGACCAGACCGAGGATT-3’ served as control (control siRNA). BAEC were transfected with 40 nM RNA according to the procedures described previously (Gonzalez et al., 2004). Twenty four to 48 h after transfection, the cells were placed in DMEM with 1% LPDS for 16 h for migration studies or in DMEM alone for cytoskeleton studies. The knockdown of Rac protein was confirmed by immunoblot analysis with anti-Rac antibody (Upstate).

Results

HDL and endothelial cell migration

To determine the effect of HDL on endothelial cell migration, BAEC monolayers were grown to near confluence and wounded by the removal of a defined region of cells with a sterile single-edge razor blade. To determine the dose response to HDL, the cells were treated with three different concentrations of HDL for 24 h in a lipoprotein deficient medium, and the number of cells migrating past the wound edge was quantitated. HDL caused a marked increase in endothelial cell migration at all three concentrations (**Figure 2-2A**). Cumulative studies revealed a 2.8, 3.6, and 4-fold increase in migration with 20, 50 and 100 μg/ml HDL, respectively (**Figure 2-2B**). Although HDL has been previously reported to induce endothelial cell migration after injury, the mechanisms regulating the migration are poorly understood (Murugesan et al., 1994).

To determine if the migration response is independent of alterations in cell proliferation, which have been previously reported to occur by HDL (Tournier et al., 1984; Chen et al., 1986), experiments were also performed in the presence of 1 mM hydroxyurea (HU). In separate experiments the prevention of cell proliferation by HU was confirmed by bromodeoxyuridine staining (data not shown). Treatment with HU did not affect HDL-induced migration (**Figure 2-3**)

indicating that HDL-induced increase in cells past the wound edge is not due to HDL-induced proliferation.

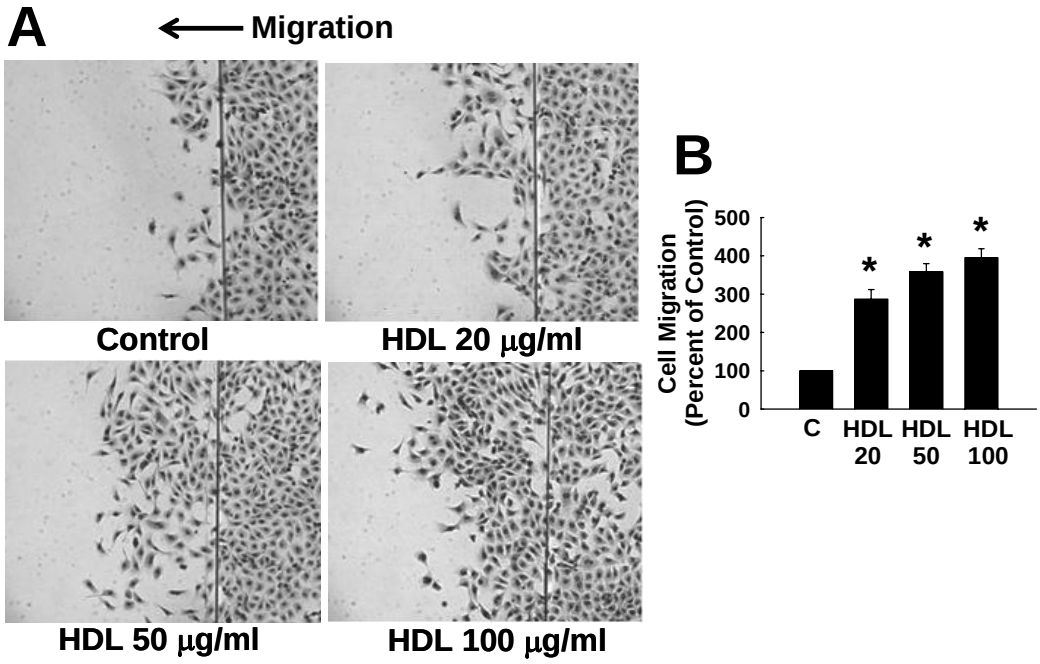

Figure 2-2: HDL induces endothelial cell migration. (A) BAEC grown to near-confluence were wounded and treated with media alone (control, C) or media plus HDL at 20, 50, or 100 μg/ml for 24 h, and migration was evaluated. (B) Migration in response to HDL was quantitated in a minimum of four fields. Values are mean±SEM, n=6. *, P<0.05 versus control.

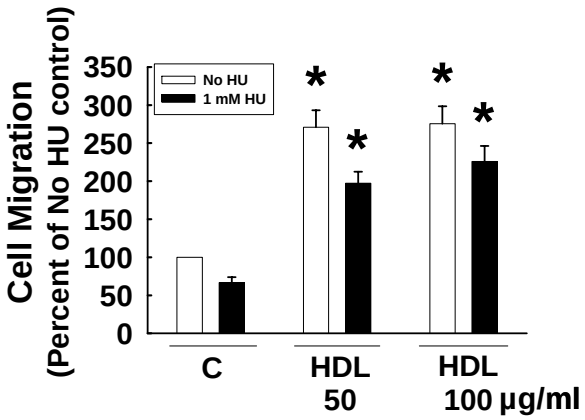

Figure 2-3: HDL-mediated migration is independent of cell proliferation. BAEC were wounded and treated with HDL +/- 1mM HU for 24 h and migration was quantitated. Values are mean±SEM, *n*=3. *, *P*<0.05 versus control.

To compare the effect of HDL with that of a well-established angiogenic factor, parallel studies were performed with HDL (50 µg/ml) and VEGF (50 ng/ml). We found that HDL-induced migration was comparable to that seen with VEGF (**Figure 2-4**).

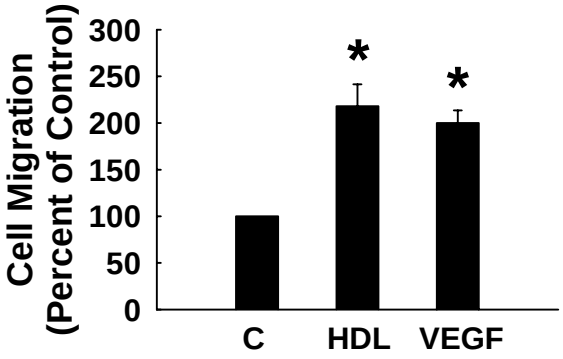

Figure 2-4: HDL mediated migration is comparable to that seen with VEGF. BAEC were wounded and treated with media alone (control, C) or media plus 50 µg/ml HDL or 50 ng/ml VEGF for 24 h and migration was quantitated. Values are mean±SEM, *n*=3. *, *P*<0.05 versus control.

In the plasma, HDL associates with various factors such as lipids and enzymes. It has been previously reported that HDL activates endothelial cell migration (Murugesan et al., 1994) and that this may be dependent upon cargo molecules on HDL such as sphingosine-1-phosphate (S-1-P) (Kimura et al., 2003). S-1-P induces endothelial cell migration in a pertussis toxin (Ptx)-sensitive manner (Kimura et al., 2003). To determine the contribution of S-1-P in HDL-induced endothelial cell migration, cells were treated with 50 μg/ml HDL or 1 μM S-1-P with or without 50 ng/ml Ptx. Although S-1-P-mediated migration was blunted by 50 ng/ml Ptx treatment, HDL-mediated migration was not affected (**Figure 2-5**), suggesting that components other than S-1-P in HDL stimulate migration of endothelial cells.

To determine the components of HDL required for the stimulation of migration, homogeneous discoidal lipoprotein Lp2A-I particles reconstituted with 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and lipid-free recombinant apoA-I were tested. Vehicle for Lp2A-I had no effect, and Lp2A-I (50 μg/ml) with POPC and apoA-I at a molar ratio of 40:1 or 80:1 did not alter migration (**Figure 2-6**). However, the addition of cholesterol to Lp2A-I yielding POPC, cholesterol and apoA-I at molar ratios of 80:5:1 caused migration which was comparable to that promoted by native HDL (50 μg/ml). The current observations indicate that HDL stimulates endothelial cell migration and that the phospholipid,

cholesterol and apoA-I components of HDL are entirely sufficient to mediate the process.

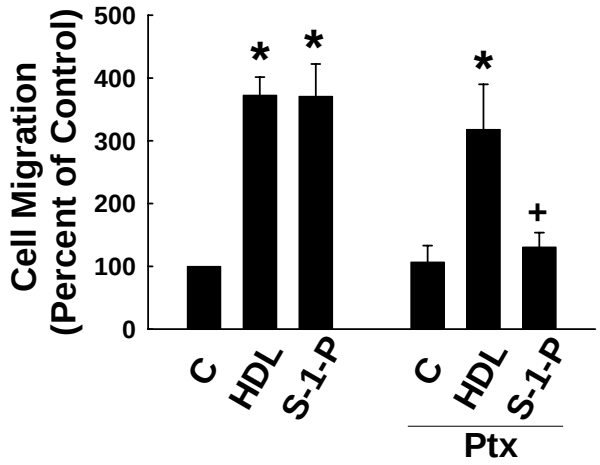

Figure 2-5: HDL-mediated endothelial cell migration is not sensitive to Ptx. BAEC were wounded and treated with media alone (control, C) or media plus 50 μg/ml HDL or 1 μM S-1-P +/- 50 ng/ml Ptx for 24 h and migration was quantitated. Values are mean±SEM, n=3. *, P<0.05 versus control, +, P<0.05 versus S-1-P alone.

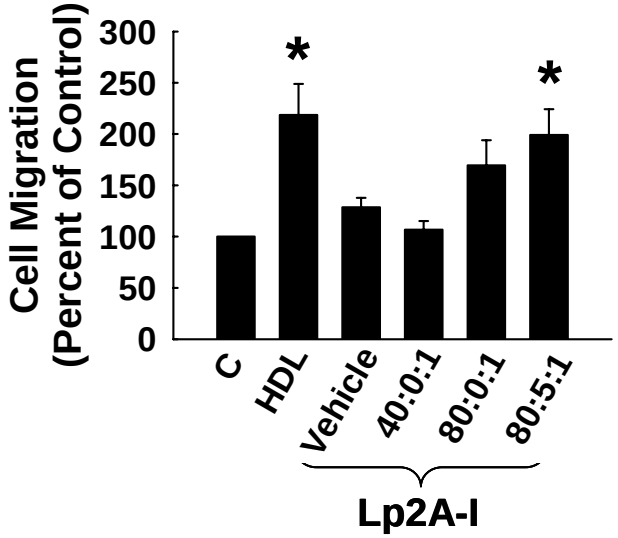

Figure 2-6: POPC, cholesterol and ApoA-I in reconstituted HDL particles are sufficient to induce endothelial cell migration. BAEC were wounded and treated with media alone (control, C), or media plus 50 μg/ml native HDL, Lp2A-I vehicle, or Lp2A-I particles with molar ratios of POPC:cholesterol:apoA-I of 40:0:1, 80:0:1 or 80:5:1 for 24 h and migration was evaluated. Values are mean±SEM, n=4. *, P<0.05 versus control.

HDL induction of lamellipodia formation

Cell migration begins with key changes in the actin cytoskeleton including the formation of lamellipodia (Ridley et al., 2003; Small et al., 2002). To assess the initiating events, we tested the effect of HDL on the endothelial cell cytoskeleton by treating BAEC with 50 μg/ml HDL for 1-30 minutes in serum-free medium and visualizing actin with phalloidin. Under control conditions, BAEC displayed multiple stress fibers and few lamellipodia. Within 1 min of HDL exposure, there was a decrease in stress fibers, an increase in lamellipodia,

and membrane ruffling (**Figure 2-7**). This effect was even more apparent at 5 min, the number of lamellipodia decreased at 15 min, and the cells appeared similar to control at 30 min of HDL treatment.

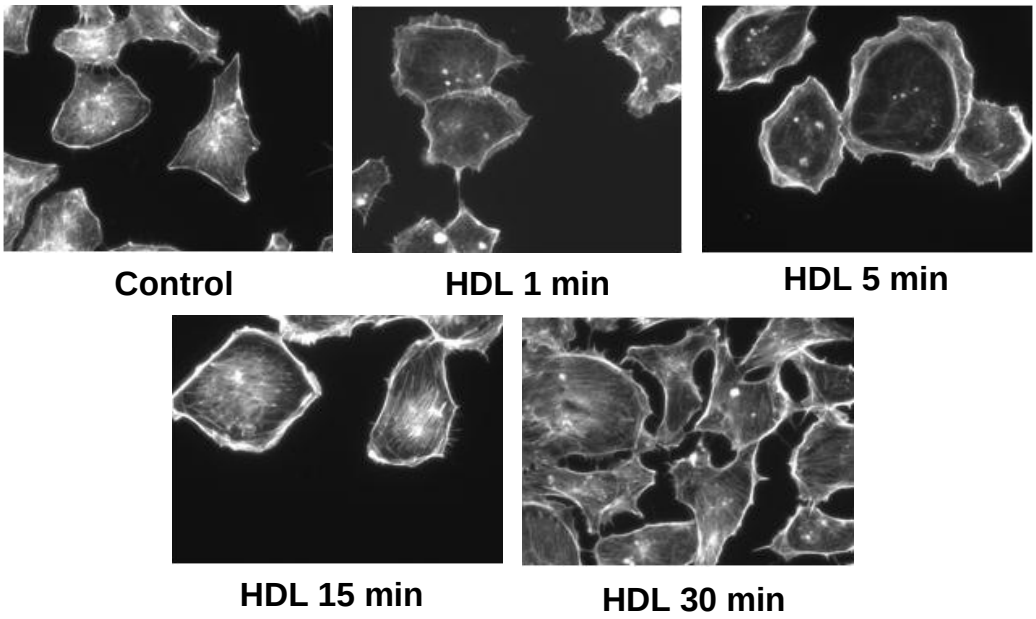

Figure 2-7: HDL induces lamellipodia formation. BAEC were treated with media alone (control) or media plus 50 μg/ml HDL for 1-30 min and changes in the actin cytoskeleton were evaluated with Alexa-568-phalloidin. Data are representative of six independent experiments.

Since the small Rho GTPase Rac mediates cell spreading and lamellipodia formation (Ridley et al., 1992), we determined if Rac GTPase is activated by HDL. BAEC were treated with 50 μg/ml HDL for 1-30 min and Rac GTPase activity was determined by assessing binding of Rac to GST-PAK. Paralleling the findings for lamellipodia formation, there was an increase in Rac GTPase activity in response to HDL within 1 min of lipoprotein exposure (**Figure**

2-8), activity was maximal at 5 min, and activity decreased after 5 min of HDL treatment. Rac activity returned to control levels at 15 min of HDL treatment. Thus, HDL causes rapid and robust lamellipodia formation in concert with Rac GTPase activation in endothelial cells.

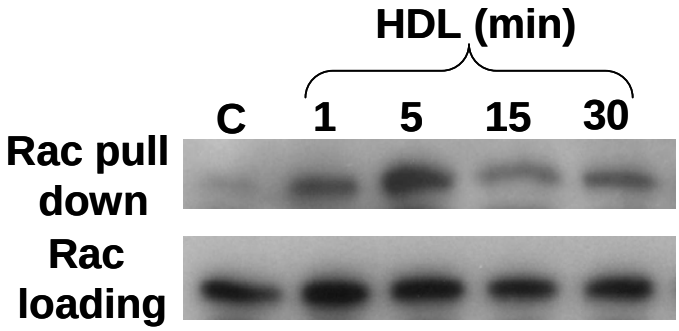

Figure 2-8: HDL induces Rac GTPase activity. BAEC were treated with media alone (control, C) or 50 μg/ml HDL for 1-30 min and Rac activity was detected. Data are representative of four independent experiments.

Role of Rac GTPase

Since Rac is an important regulator of cell migration and lamellipodia formation in many cell types (Hu et al., 2002; Nobes and Hall, 1995; Nobes and Hall, 1999) and it is activated by HDL, the role of Rac was tested in HDL-induced endothelial cell migration and lamellipodia formation using Rac knockdown studies. In the recent years, RNA interference has become a widely used method of specifically knocking down a certain gene product. BAEC were

transfected with 40 nM control double-stranded (ds) siRNA or dsRNA targeting the open reading frame of bovine Rac1 (Gonzalez et al., 2004). Knockdown of Rac protein was observed 48 hours after transfection with Rac siRNA (**Figure 2-9A**). BAEC transfected with control siRNA migrated in response to 50 μg/ml HDL. However, cells transfected with Rac siRNA did not migrate in response to HDL (**Figure 2-9B**). The role of Rac in HDL-induced lamellipodia formation was also tested in BAEC after transfection with control siRNA or Rac siRNA. Control siRNA-transfected cells had an increase in lamellipodia formation after 5 min of 50 μg/ml HDL treatment whereas cells transfected with Rac siRNA did not respond to HDL (**Figure 2-9C**). These data indicate that HDL-induced migration and lamellipodia formation require Rac GTPase.

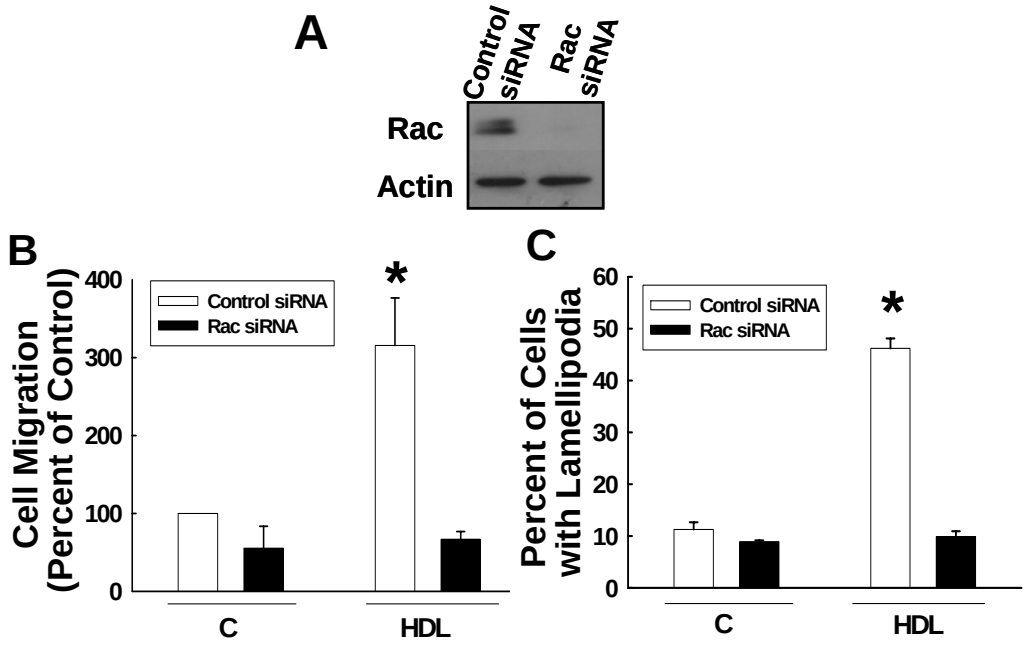

Figure 2-9: HDL-mediated migration and lamellipodia formation require Rac GTPase. (A) BAEC were transfected with 40 nM control or Rac siRNA and harvested after 48 h. Lysates were subjected to immunoblot analysis for Rac or actin. (B) BAEC were transfected with 40 nM control or Rac siRNA. Cells were wounded and treated with media alone (control, C) or 50 μg/ml HDL and migration was quantitated. (C) BAEC were transfected with 40 nM control or Rac siRNA. Cells were treated with media alone (control, C) or 50 μg/ml HDL and percent of cells with lamellipodia was quantitated. For B and C, values are mean±SEM, n=3. *, P<0.05 versus control.

Conclusions

Factors such as cytokines, disturbed shear stress, reactive oxygen species and physical stress cause vascular injury leading to endothelial dysfunction and compromise of the endothelial barrier. Efficient endothelial repair after injury restores endothelial integrity. In addition to inducing NO production, we observed that HDL promotes endothelial cell migration after injury as seen in monolayers of BAEC *in vitro*. HDL caused an increase in the number of cells migrating past the wound edge and this effect was independent of effects on cell proliferation as seen by migration experiments performed in the presence of hydroxyurea. HDL-induced migration was comparable to that seen with VEGF and independent of cargo molecules such as S-1-P. Although Kimura et al observed an increase in HDL-mediated migration due to S-1-P, the treatment conditions in their experiments were different compared to the ones used in these experiments (Kimura et al., 2003). They used 100 ng/ml Ptx and pre-treated cells for 24 hours before the addition of HDL or S-1-P. Under these conditions, we observed that basal migration was completely blunted by Ptx. This is not a reliable measurement of an HDL-mediated Ptx-sensitive pathway since the basal migration is blunted. Therefore, in the experiments shown in Figure 2-5, we used a concentration of Ptx which did not affect basal migration. Although this concentration blunted S-1-P-mediated migration, HDL-mediated migration was not affected. In experiments using reconstituted particles with POPC, apoA-I and

cholesterol, increased POPC and the addition of cholesterol to the particles caused an increase in endothelial cell migration comparable to that seen with 50 μg/ml HDL. In a recent study, the addition of cholesterol to endothelial cells resulted in stimulation of migration due to the increase in microviscosity of the plasma membrane (Ghosh et al., 2002). Treatment of cells with growth factors such as VEGF also increased membrane microviscosity and Rac activity resulting in stimulation of cell motility. Therefore, the addition of cholesterol to Lp2A-I particles may induce cell migration due to an increase in membrane viscosity. Further studies are required to test if HDL or cholesterol-containing Lp2A-I particles induce migration by causing increases in membrane viscosity and if the presence of cholesterol is required for Rac activation.

The initiating event in cell migration is cytoskeletal rearrangement. We observed that HDL causes lamellipodia formation within 5 min of treatment. The lamellipodia extensions decrease after 5 min and the cells appear similar to control cells around 15 min of treatment. The cycle of lamellipodia formation and retraction and subsequent stress fiber formation is required for cell movement to occur (Ridley, 2001). The main molecular machinery controlling cytoskeletal rearrangements is the Rho family of GTPases. Lamellipodia formation is initiated by activation of Rac GTPase (Nobes and Hall, 1995). We observed that HDL causes robust Rac activation within minutes and this corresponds to the lamellipodia formation. Both lamellipodia formation and Rac activation decrease

after 15 minutes of HDL treatment indicating a negative regulation to switch off the signaling to Rac. This is consistent with the mechanistically separate steps of migration which suggest a cyclic pathway involving lamellipodia extension, formation of new adhesions, cell contraction, and tail retraction (Ridley, 2001). The re-appearance of stress fibers after 30 minutes of HDL treatment is indicative of the cell entering another stage of migration. Rac is also required for HDL-induced endothelial cell migration and lamellipodia formation as seen in the studies of Rac knockdown. These results indicate that HDL induces endothelial cell migration and lamellipodia formation in association with Rac activation and that Rac GTPase is required for these responses.

CHAPTER THREE: Role of kinases, eNOS and SR-BI in HDL induced migration and lamellipodia formation

Introduction

The regulation of signaling initiated by an agonist to promote endothelial cell migration involves multiples factors such as Rho GTPases, kinases, and other cytoskeletal proteins. In previous studies, HDL caused an increase in eNOS activity in endothelial cells. This activation requires the activities of Src kinases, and the sequential activation of PI3K which in turn stimulates Akt kinase and MAPK. Akt kinase phosphorylates eNOS on serine 1179 which allows for eNOS activation. Although MAPK is not required for eNOS phosphorylation, it is necessary for the activation of eNOS by HDL via an unknown mechanism (Mineo et al., 2003).

In order to determine the mechanism by which HDL signals to induce endothelial cell migration and lamellipodia formation, we initially tested the role of the kinases known to be involved in HDL-mediated eNOS activation. Src family kinases, PI3K and MAPK have been previously shown to be involved in endothelial cell migration in response to various agonists (Abu-Ghazaleh et al., 2001; Goetze et al., 2002; Zhang et al., 2004) and all three regulate cytoskeletal proteins which are required for cell movement. In addition to the kinases, eNOS regulates endothelial cell migration and angiogenesis (Chen et al., 2004;

Kawasaki et al., 2003). Since HDL stimulates kinases leading to the activation of eNOS, we tested the role of the kinases and eNOS in HDL-induced endothelial cell migration and lamellipodia formation.

SR-BI is enriched in the caveolae membrane of BAEC (**Figure 3-1**) and is required for HDL-induced activation of eNOS (Yuhanna et al., 2001). In addition to determining the role of the kinases and eNOS, we determined if SR-BI is required for HDL-induced endothelial cell migration and lamellipodia formation.

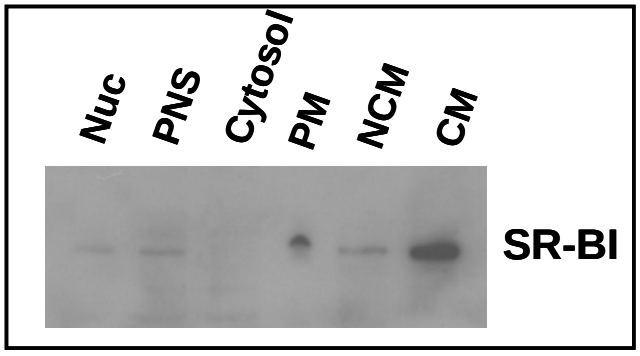

Figure 3-1: SR-BI is enriched in the caveolae membrane (CM) of BAEC. PNS: post-nuclear supernatant; NCM: non-caveolae membrane. After isolation of cell fractions, the lysates were subjected to gel electrophoresis and immunoblot analysis.

Methods

Endothelial Cell Migration Assay: Migration of BAEC in response to various treatments was evaluated as mentioned in the methods section of Chapter Two. In studies to determine the role of the kinases or eNOS, cells were pre-treated with pharmacological inhibitors at concentrations of 500 nM PP2, 2 μM LY 294002, 1 μM PD 98059 (Calbiochem), or 2 mM Nω-Nitro-$_L$-arginine methyl ester (L-NAME) (Sigma) for 30 min before the addition of 50 μg/ml HDL. Results were confirmed in a minimum of three independent experiments.

Cytoskeletal Changes in Endothelial Cells: The changes in the cytoskeleton were determined as mentioned in the methods section of Chapter Two. In studies to determine the role of the kinases or eNOS, cells were pre-treated with 10 μM PP2, 10 μM PD 98059, 20 μM LY 294002, or 2 mM L-NAME for 30 min before the addition of 50 μg/ml HDL for 5 min. The percent of cells with lamellipodia was quantified in a minimum of 100 total cells. For statistical analyses, values from a minimum of three independent experiments were combined.

Rac Activity Assay: Rac activity was determined as described in the methods section of Chapter Two. In studies to determine the role of the kinases, cells were pre-treated with 10 μM PP2, 10 μM PD 98059 or 20 μM LY 294002 for 30 min

and treated with 50 μg/ml HDL for 5 min. After treatment, Rac activity was measured. Results were confirmed in four independent experiments.

Migration of MFLM 91U: Migration of MFLM 91U, an immortalized mouse endothelial cell line (provided by A. Akeson, Children's Hospital Medical Center, Cincinnati, OH), was evaluated as mentioned in the methods section of Chapter Two for BAEC. In studies to determine the role of SR-BI, the cells were pre-treated with 1:1000 dilution of anti-SR-BI antibody (Novus Biologicals), or normal non-specific IgG (Santa Cruz) for 1 h before the addition of 50 μg/ml HDL for 24 h.

siRNA Preparation and Transfection: A dsRNA with sequence 5'-UCCGGAGCCAAGAGAAUGTT-3' was designed to target the open reading frame of bovine SR-BI (gi: 2429347) or 5'-UGCGUUUCCUGGAGAAUAU-3' was designed to target the open reading frame of bovine Rac1 (Gonzalez et al., 2004). A dsRNA with sequence 5'-AGUUAGACCAGACCGAGGATT-3' served as control (control siRNA). BAEC were transfected with 40 nM RNA as described previously (Gonzalez et al., 2004). Twenty four to 48 h after transfection, the cells were placed in DMEM with 1% LPDS for 16 h for migration studies or in DMEM alone for cytoskeleton studies, Rac activity assays, or phosphorylation studies. The decrease in SR-BI or Rac protein was confirmed

by immunoblot analysis with anti-SR-BI antibody (Acton et al., 1996) (provided by Dr. H. Hobbs, UT Southwestern) or anti-Rac antibody (Upstate), respectively. For phosphorylation studies, cells were treated with 50 μg/ml HDL for 5 or 10 min. Cells were harvested and lysates were subjected to SDS-PAGE using antibodies to phospho-Src (Cell Signaling), c-Src (Santa Cruz), phospho-Akt, Akt, phospho-MAPK (Cell Signaling) and MAPK (Upstate).

Results

Role of kinase activation

To further delineate the mechanisms by which HDL stimulates endothelial cell migration, we determined whether the process entails the kinases which have been implicated in HDL-mediated eNOS activation. To test the role of Src kinases, the impact of the pharmacologic inhibitor PP2 was determined. Whereas treatment with 500 nM PP2 did not affect basal migration, HDL-stimulated migration was blunted by 72% by PP2 (**Figure 3-2A**). The negative control compound for PP2, PP3, had no effect on migration (data not shown). The role of PI3 kinase was assessed using LY 294002. Treatment with 2 μM LY294002 did not affect basal migration. However, HDL-stimulated migration was fully attenuated (**Figure 3-2B**). PD 98059 (1 μM) was used to inhibit the activity of MAP kinase kinase, or MEK, which resides immediately upstream of MAPK. The inhibition of MEK did not alter basal migration, but HDL-induced migration was attenuated by 84% (**Figure 3-2C**). Therefore, HDL-induced endothelial cell migration requires the activation of Src family kinases, PI3K, and MAPK.

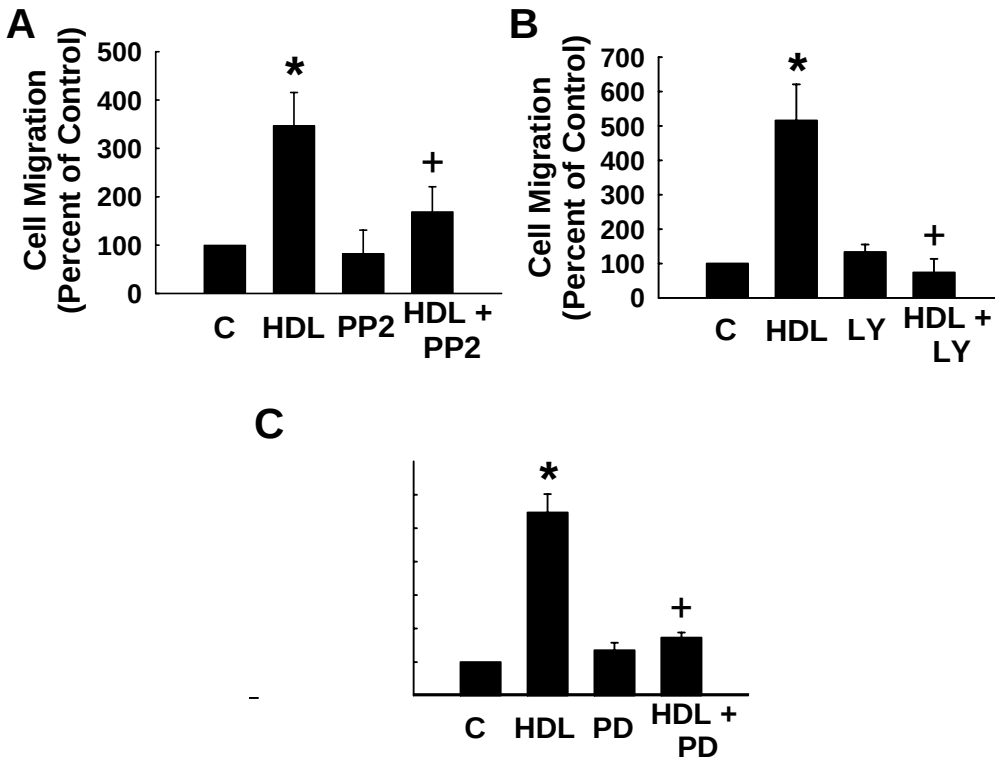

Figure 3-2: HDL-mediated migration requires the activities of Src family kinases, PI3K and MAPK. BAEC were treated with HDL +/- 500 nM PP2 (A), 2 μM LY 294002 (B), or 1 μM PD 98059 (C) for 24 h and migration was quantitated. Values are mean±SEM, *n*=3-8. *, *P*<0.05 versus control, +, *P*<0.05 versus HDL alone.

The roles of Src kinases, PI3K and the MAPK cascade in initial HDL-induced lamellipodia formation were also evaluated. BAEC pretreatment with PP2 (10 μM) had no effect on the cytoskeleton at baseline. However, the 14-fold increase in lamellipodia formation with HDL (50 μg/ml for 5 min) (**Figure 3-3A**) was blunted by 87% by PP2. The negative control compound PP3 had no effect on lamellipodia formation (data not shown).

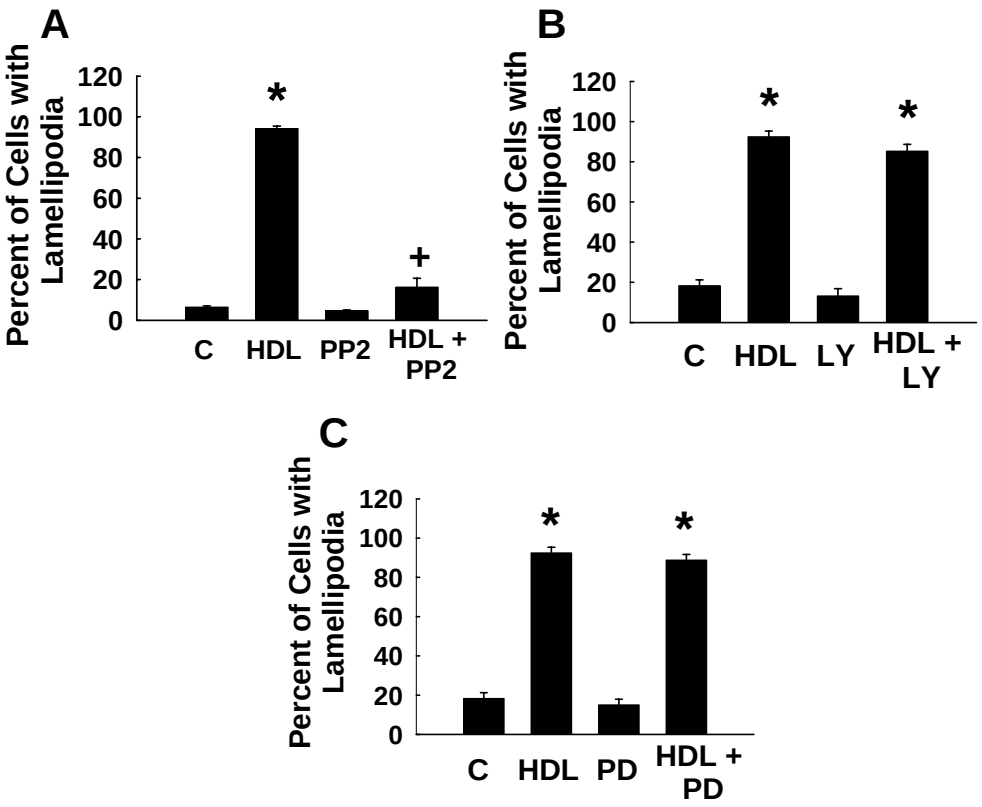

Figure 3-3: HDL-induced lamellipodia formation requires Src family kinases, but not PI3K and MAPK. BAEC were treated with HDL +/- 10 μM PP2 (A), 20 μM LY 294002 (B), or 10 μM PD 98059 (C) and lamellipodia formation was quantitated. Values are mean±SEM, *n*=3-8. *, *P*<0.05 versus control, +, *P*<0.05 versus HDL alone.

In studies of PI3K involvement using LY294002 (20 μM), the compound did not modify lamellipodia formation in response to HDL (**Figure 3-3B**). PD98059 (10 μM) antagonism of MEK activity also had no effect (**Figure 3-3C**). The efficacy of LY294002 and PD98059 was confirmed in additional studies

revealing decreases in HDL-stimulated Akt and MAPK phosphorylation, respectively (data not shown). When considered along with the observations for cell migration, these findings indicate that Src family kinases, which reside upstream of PI3K and MAPK in HDL signaling to eNOS (Mineo et al., 2003), modulate both immediate cytoskeletal responses and ultimate cell migration with HDL. In contrast, PI3K and MAPK are not required for initial actin rearrangement but are necessary for cell migration in response to HDL.

To determine the role of these kinases in HDL-induced Rac activation, BAEC were treated with PP2, LY294002 or PD98059. Treatment with PP2 (10 μM) to assess the role of Src family kinases did not alter basal Rac activity. However, HDL-induced Rac activation was blunted by 67% in response to PP2 treatment (**Figure 3-4A**). Treatment with 20 μM LY294002 to determine PI3K involvement did not affect basal Rac activity but blunted HDL-mediated Rac activation by 86% (**Figure 3-4B**). To determine the role of MAPK, BAEC were treated with 10 μM PD98059. Basal Rac activity was not affected by PD98059 treatment, but HDL-induced Rac activation was attenuated by 82% (**Figure 3-4C**). Therefore, Src family kinases, PI3K and MAPK are required for HDL-induced Rac activity.

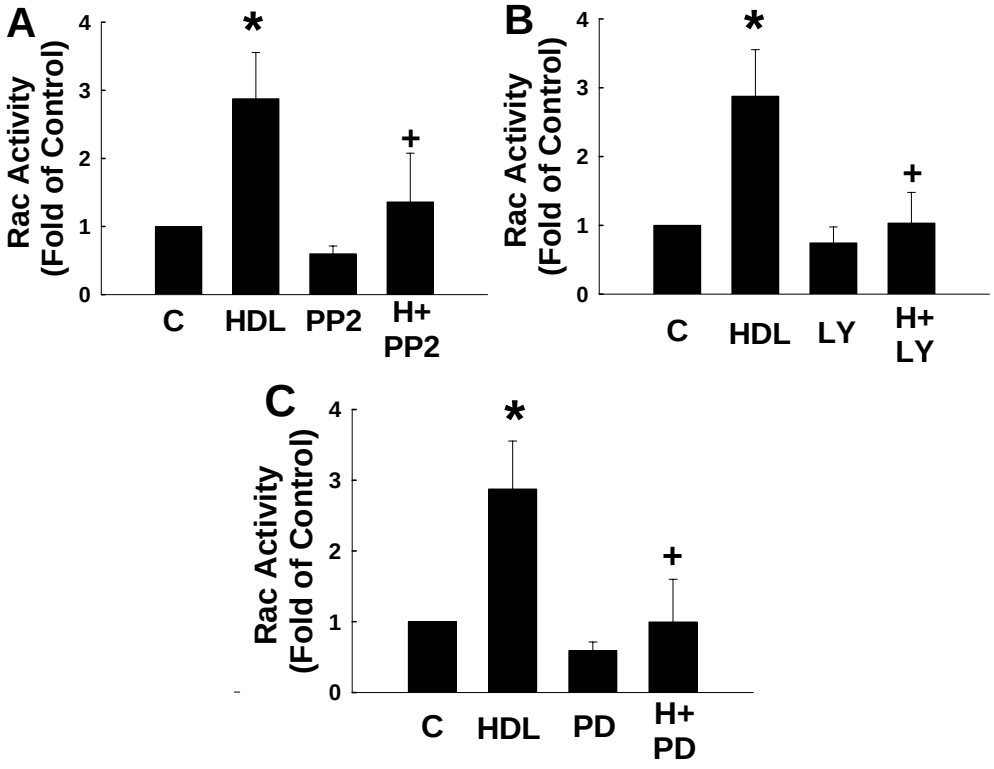

Figure 3-4: HDL-induced Rac activation requires Src family kinases, PI3K and MAPK. BAEC were treated with HDL +/- 10 μM PP2 (A), 20 μM LY 294002 (B), or 10 μM PD 98059 (C) and Rac activation was quantitated. Values are mean±SEM, *n*=3-8. *, *P*<0.05 versus control, +, *P*<0.05 versus HDL alone.

To verify that Rac is downstream of the kinases, BAEC were transfected with control siRNA or Rac siRNA and HDL-induced phosphorylation of Src, PI3K and MAPK was tested. HDL (50 μg/ml) caused phosphorylation of Src, PI3K and MAPK within 5 min of treatment in control siRNA and Rac siRNA-transfected cells (**Figure 3-5**). Therefore, Rac is not required for HDL-mediated

phosphorylation of Src, PI3K or MAPK and is downstream of these kinases in the HDL-signaling pathway.

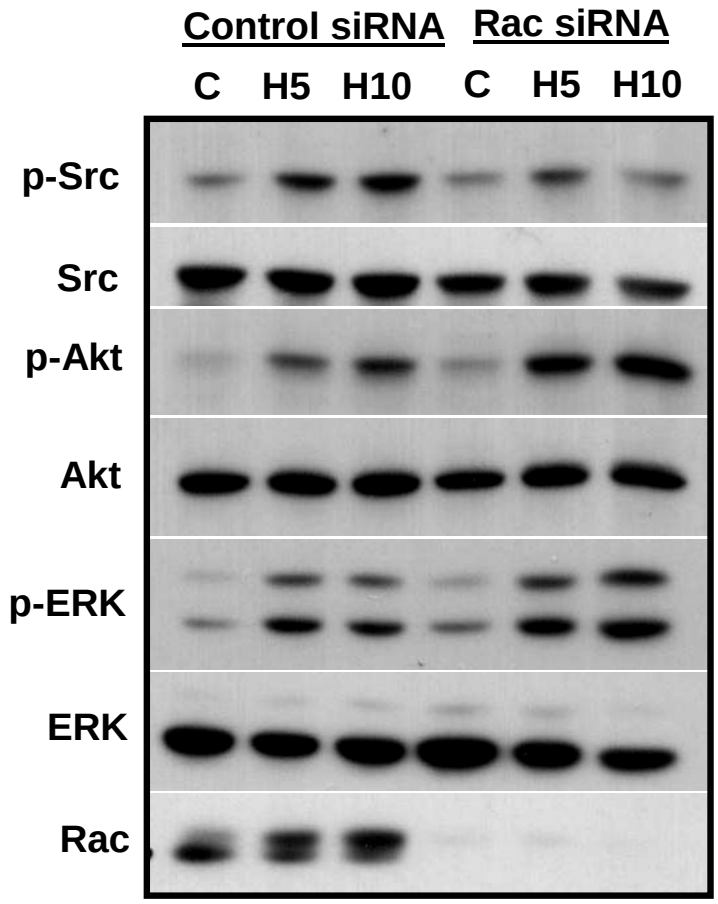

Figure 3-5: HDL-induced phosphorylation of Src, PI3K and MAPK does not require Rac. BAEC were transfected with 40 nM control siRNA or Rac siRNA, and the phosphorylation of Src, PI3K and MAPK induced by HDL (H, 50 μg/ml for 5 or 10 min) were detected by immunoblot analysis using phospho-specific antibodies. Results shown were confirmed in 4 independent experiments.

Role of eNOS

NO promotes endothelial cell migration and angiogenesis (Chen et al., 2004; Kawasaki et al., 2003), and numerous stimuli including VEGF induce these processes via the activation of eNOS (Ashton and Ware, 2004; Shizukuda et al., 1999). Since HDL causes potent eNOS stimulation (Yuhanna et al., 2001), we determined if the enzyme is required for HDL-mediated endothelial cell migration. BAEC responses to HDL (50 μg/ml) were tested in the absence or presence of the NOS antagonist L-NAME (2 mM). Treatment with L-NAME did not affect basal migration, and HDL-induced endothelial cell migration was also not attenuated (**Figure 3-6A**). In contrast, VEGF-stimulated migration was predictably blocked by NOS antagonism (**Figure 3-6B**). We also tested the role of eNOS in HDL-induced alterations in the actin cytoskeleton (50 μg/ml HDL for 5 min) (**Figure 3-6C**). L-NAME did not alter the basal number of cells displaying lamellipodia, and lamellipodia formation in response to HDL was not changed. The efficacy of L-NAME was verified by confirming the inhibition of eNOS enzymatic activity (data not shown). These findings indicate that in contrast to the mechanisms of action of multiple known stimuli including VEGF (Ashton and Ware, 2004; Shizukuda et al., 1999), the promotion of endothelial cell migration by HDL is NO-independent.

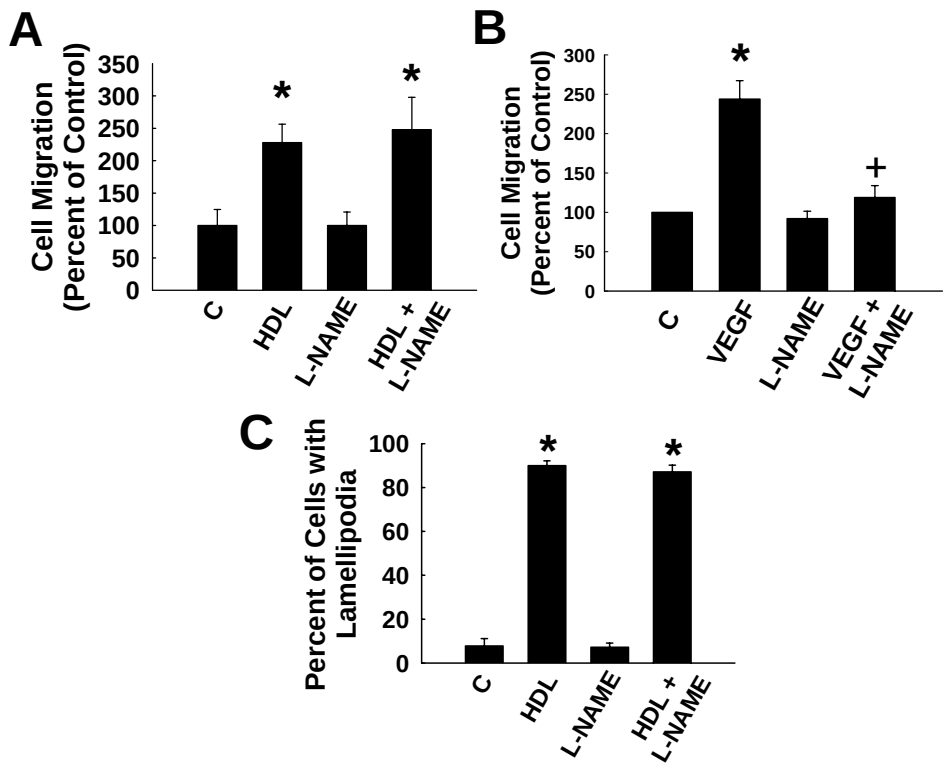

Figure 3-6: HDL-induced endothelial cell migration and lamellipodia formation are independent of eNOS. BAEC grown to near-confluence were wounded and treated with media alone (control, C) or media plus 50 μg/ml HDL (A) or 50 ng/ml VEGF (B) for 24 h in the absence or presence of 2 mM L-NAME and migration was evaluated. (C) BAEC were treated with media alone (control, C) or media plus 50 μg/ml HDL for 5 min in the absence or presence of 2 mM L-NAME, and the percent of cells with lamellipodia was quantified. Values are mean±SEM, n=3-4. *, $P< 0.05$ versus control, +, $P<0.05$ versus VEGF alone.

Role of SR-BI

The high affinity HDL receptor SR-BI resides within caveolae/lipid rafts on the plasma membrane of endothelial cells (Uittenbogaard et al., 2000). In order to determine the role of SR-BI in HDL-mediated endothelial cell migration

and lamellipodia formation, we initially used the extracellular blocking antibody against SR-BI. This antibody inhibits the binding of HDL to SR-BI and prevent cholesterol efflux to HDL (Gu et al., 2000). Since the antibody recognizes the mouse SR-BI protein, we used immortalized mouse endothelial cells from embryonic lung mesenchyme, MFLM 91U (Akeson et al., 2000). The MFLM 91U cells migrated in response to 50 μg/ml HDL after wounding and treatment for 24 h (**Figure 3-7A**). In order to determine the role of SR-BI in HDL-mediated migration, the cells were treated with the anti-SR-BI antibody at 1:1000 dilution in the absence or presence of HDL and migration was evaluated. Treatment with a non-specific IgG did not have an effect on migration. Interestingly, the antibody did not block HDL-mediated migration. In fact, the antibody alone increased migration to a degree similar to that of HDL and modestly increased migration in the presence of HDL. MFLM 91U cells were also used to test the role of SR-BI in HDL-mediated lamellipodia formation. The cells displayed increased lamellipodia formation in response to HDL and this increase was not affected in the presence of non-specific IgG (**Figure 3-7B**). In contrast to the results of the migration experiment, the SR-BI antibody blunted HDL-mediated lamellipodia formation, but increased basal lamellipodia formation in the absence of HDL. Due to the increase in basal migration and lamellipodia formation in the presence of the antibody, the results of these experiments are inconclusive. One possible explanation for these findings is that the antibody binding to SR-BI may

cause a "ligand-like" effect resulting in increased migration and lamellipodia formation.

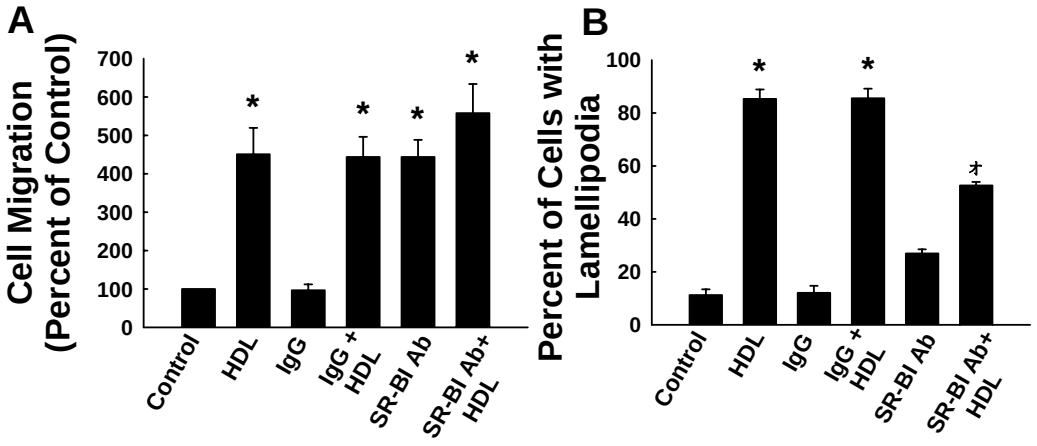

Figure 3-7: Effect of SR-BI blocking antibody on HDL-induced migration and lamellipodia formation (A) MFLM 91U cells were wounded and treated with media alone (control), non-specific IgG, or 50 μg/ml HDL in the absence or presence of anti-SR-BI antibody. Migration was evaluated 24 h later. Values are mean±SEM, n=6. *, P< 0.001 versus control. Data is representative of 3 independent experiments. (B) MFLM91U cells were treated with media alone (control) non-specific IgG, or 50 μg/ml HDL for 5 min in the absence or presence of anti-SR-BI antibody and lamellipodia formation was evaluated. Values are mean±SEM, n=4-5. *, P< 0.001 versus control, +, P<0.001 versus HDL alone.

A second approach was used to determine if SR-BI plays a role in HDL mediated endothelial cell migration. We employed the RNAi method to study the role of SR-BI by using dsRNA targeting SR-BI (SR-BI siRNA) or control siRNA (40 nM each) which were transfected into BAEC. Effective knockdown of SR-BI

protein by SR-BI siRNA but not control siRNA was observed by immunoblot analysis (**Figure 3-8**).

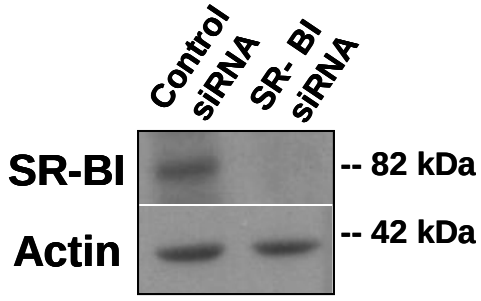

Figure 3-8: Knockdown of SR-BI after SR-BI siRNA transfection. BAEC were transfected with 40 nM control or SR-BI siRNA and the cells were harvested 48 h later.

Forty eight h after transfection with control siRNA or SR-BI siRNA, BAEC were wounded and treated with 20 or 50 μg/ml HDL for 24 h. The cells transfected with control siRNA displayed an 8.5-fold increase in migration with HDL (**Figure 3-9A**). In contrast, cells transfected with SR-BI siRNA did not migrate in response to HDL. Knockdown of SR-BI in BAEC was also used to determine the role of SR-BI in HDL-mediated changes in the actin cytoskeleton. In cells transfected with control siRNA, there was a marked 5.3-fold increase in lamellipodia formation in response to 50 μg/ml HDL treatment for 5 min (**Figure 3-9B**). In contrast, after knockdown of SR-BI, lamellipodia formation did not occur with HDL treatment.

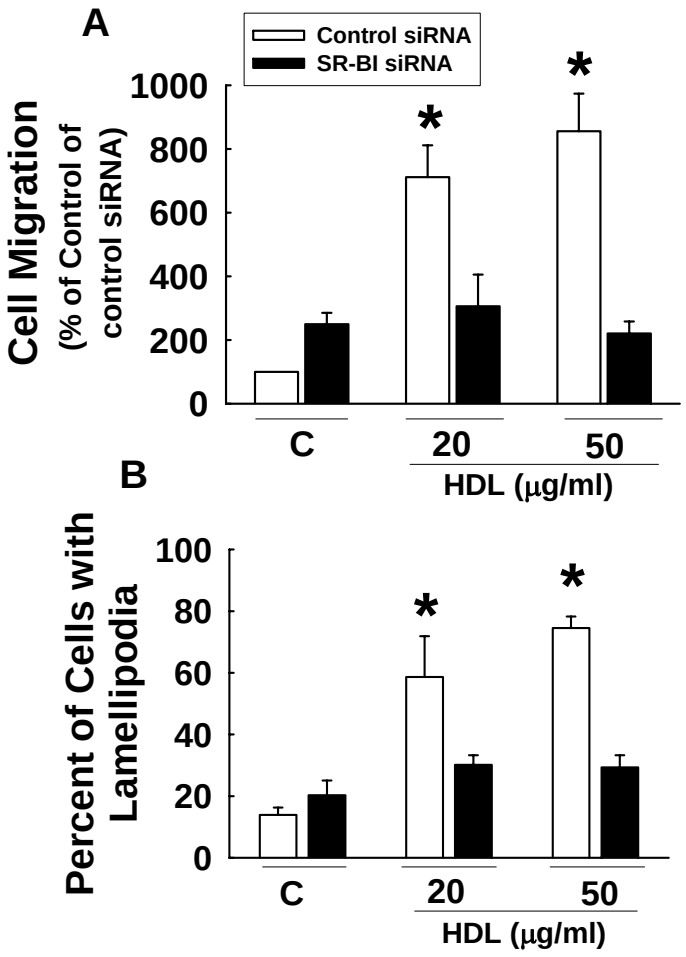

Figure 3-9: HDL-induced migration and lamellipodia formation require the presence of SR-BI. (A) BAEC transfected with 40 nM control siRNA or SR-BI siRNA were wounded and treated with media alone (control, C) or media plus 50 μg/ml HDL for 24 h, and migration was evaluated. Values are mean±SEM, n=11. *, P< 0.001 versus control. (B) BAEC transfected with 40 nM control siRNA or SR-BI siRNA were treated with media alone (control, C) or media plus 50 μg/ml HDL for 5 min and the percent of cells with lamellipodia was quantified. Values are mean±SEM, n=4. *, P< 0.001 versus control.

To determine if SR-BI is required for HDL-induced Rac activity, SR-BI was knocked down using SR-BI siRNA and HDL-induced Rac activation was determined. In cells transfected with control siRNA, treatment with 50 μg/ml HDL for 5 min caused an increase in the amount of GST-PAK-bound Rac (**Figure 3-10**). However, after knockdown of SR-BI, HDL-induced Rac activation was blunted compared to control siRNA-treated cells. This suggests that SR-BI is required for HDL-induced Rac activity. These cumulative results indicate that SR-BI is critically involved in the activation of endothelial cell migration, lamellipodia formation, and Rac activation induced by HDL.

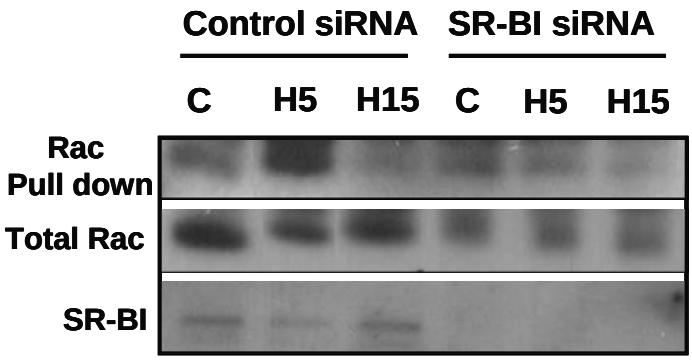

Figure 3-10: SR-BI is required for HDL-induced Rac activity. BAEC transfected with 40 nM control siRNA or SR-BI siRNA were treated with media alone (control, C) or media plus 50 μg/ml HDL for 5 min and Rac activity and SR-BI abundance were determined. Results shown are representative of 4 independent experiments.

Conclusions

HDL induces eNOS activity in endothelial cells by activating Src family kinases, PI3K and MAPK. The role of these kinases was also tested in HDL-induced endothelial cell migration and lamellipodia formation. We determined that HDL-mediated migration requires the activities of all three kinases. This was seen in migration experiments where pharmacological inhibitors of the kinases did not blunt basal migration, but inhibited HDL-induced migration of BAEC. In studies of HDL-mediated lamellipodia formation, Src family kinases were required whereas PI3K or MAPK were not required for HDL-induced lamellipodia formation. In contrast to the lamellipodia studies, the involvement of all three kinase pathways was observed for HDL-mediated Rac activity using pharmacological inhibitors.

Since cell migration is a complex phenomenon entailing not only lamellipodia formation but also cell adhesion to matrix contacts, contraction, and release from matrix contacts (Larsen et al., 2003), we postulate that HDL-related PI3K and MAPK activation modify signaling events required for the latter processes, such as focal adhesion kinase activation and myosin light chain phosphorylation (Jung et al., 2004; Klemke et al., 1997). The discrepancy in the migration, lamellipodia formation and Rac activity readouts may also stem from the difference in sensitivity of the three assays. The fraction of active Rac in the

cells is small compared to total Rac. Whereas lamellipodia formation can be detected easily even with small changes in the cell morphology, the Rac activity assay detects only a small fraction of active Rac. Therefore, small amounts of active PI3K or MAPK in the cells which are not altered by the inhibitors, may allow HDL-mediated lamellipodia formation and detection by actin staining. A third possibility for the absence of inhibition by LY294002 or PD98059 for lamellipodia formation is activation of an unknown process which is downstream of Src family kinases and upstream of Rac.

The studies using the extracellular blocking antibody against SR-BI to determine the role of SR-BI in HDL-migration resulted in stimulation of migration by the antibody alone. An additive effect was observed by the antibody in the presence of HDL. These data indicate that antibody binding to SR-BI may cause a "ligand-like" effect resulting in increased migration of endothelial cells. This hypothesis can be tested by assessing phosphorylation/activation of the HDL signaling pathway in response to the antibody. One way in which the antibody may increase signaling by SR-BI is via increased SR-BI dimerization. SR-BI dimerization has been observed in cells and tissues involved in selective cholesteryl ester uptake (Landschulz et al., 1996; Reaven et al., 2004; Williams et al., 2000), implicating the role of dimeric forms of SR-BI in cholesterol flux. However, the role of SR-BI dimerization in HDL-mediated signaling or cholesterol efflux is unclear. Further studies are required to delineate the role of

SR-BI dimerization in HDL-mediated signaling and cholesterol flux. Due to the inconclusiveness of these results, we used an alternative approach of RNAi against SR-BI, which is more specific and reliable compared to the antibody strategy. SR-BI knockdown in endothelial cells by the dsRNA blunted migration, lamellipodia formation and Rac activation in response to HDL, whereas control siRNA did not. These results place SR-BI most proximal to HDL in the signaling pathway and indicate the requirement of SR-BI for HDL-mediated migration, lamellipodia formation and Rac activation.

CHAPTER FOUR: Role of ApoA-I and SR-BI in reendothelialization *in vivo*

Introduction

HDL-mediated endothelial cell migration *in vitro* has implications for endothelial repair *in vivo*. Several models of endothelial injury and repair have been established by multiple groups. One such injury model entails electric injury (thermal injury) of the femoral or carotid artery resulting in denudation of the endothelial cells (Brouchet et al., 2001; Carmeliet et al., 1997). This injury model can be used to detect subsequent neointima formation or reendothelialization. Although the wire injury model has been widely used, the thermal injury model allows for reliable quantitation of the area of remaining denudation by evaluation of Evan's blue incorporation in the region of denudation.

In order to determine the role of HDL and SR-BI in reendothelialization *in vivo*, we used mice lacking apoA-I and SR-BI. ApoA-$I^{-/-}$ mice have an 83% decrease in circulating HDL levels. The role of apoA-I in preventing atherosclerosis is exemplified in apoB transgenic/apoA-$I^{-/-}$ or $LDLR^{-/-}$/apoA-$I^{-/-}$ mice which are more susceptible to atherosclerosis compared to apoB transgenic or $LDLR^{-/-}$ mice (Hughes et al., 1997; Moore et al., 2003; Voyiaziakis et al., 1998). Several studies have indicated that supplementing mice with an apoA-I-containing adenovirus increases apoA-I and HDL levels in the plasma. Liver-directed gene transfer of an apoA-I-containing adenovirus into mice of

atherogenic backgrounds such as apoE$^{-/-}$ or LDLR$^{-/-}$ mice leads to decreased neointima formation after wire injury and decreased atherosclerotic lesions in the aortic arch and aortic root (De Geest et al., 1997; Tangirala et al., 1999).

Similar to apoA-I, SR-BI is protective against atherogenesis as observed in studies of SR-BI$^{-/-}$ mice. SR-BI deficiency in mice leads to increased lipid deposition in the aorta after a high fat diet compared to SR-BI wild type mice (Van Eck et al., 2003). SR-BI$^{-/-}$ mice crossed with apoE$^{-/-}$ mice have increased plasma cholesterol and an accelerated onset of atherosclerosis compared to SR-BI$^{-/-}$ or apoE$^{-/-}$ mice. At 4-7 weeks of age, no atherosclerotic lesions are present in apoE$^{-/-}$ mice. However, SR-BI$^{-/-}$/apoE$^{-/-}$ mice contain lesions in the aortic root, aortic sinus and coronary arteries (Trigatti et al., 1999).

Although these studies indicate an atheroprotective role of apoA-I and SR-BI in mice, the underlying mechanisms are unclear. Using the model of thermal injury in apoA-I$^{-/-}$ and SR-BI$^{-/-}$ mice, we determined the role of these proteins in reendothelialization *in vivo*. In addition, we increased the apoA-I and HDL levels in apoA-I$^{-/-}$ mice to determine if reendothelialization can be rescued after normalizing the levels of the lipoprotein.

Thickening of the intima or the media of the arterial wall can occur after an injury such as wire injury or a balloon angioplasty. The increase in intimal thickening due to neointima formation occurs in mice of atherogenic backgrounds and can be rescued by apoA-I supplementation (De Geest et al., 1997). In our

studies, we determined if apoA-I$^{-/-}$ and SR-BI$^{-/-}$ mice are prone to neointima formation after thermal endothelial injury.

Methods

Carotid Artery Reendothelialization: Carotid artery reendothelialization was studied following perivascular electric injury (Brouchet et al., 2001) in 12-16 week-old male C57BL/6 apoA-$I^{+/+}$ versus C57BL/6 apoA-$I^{-/-}$ mice (Jackson Labs), and in SR-$BI^{+/+}$ versus SR-$BI^{-/-}$ mice (provided by Dr. H. Hobbs, UT Southwestern and Dr. M. Krieger, Massachusetts Institute of Technology). Additional experiments were performed in 30-35 week-old SR-$BI^{+/+}$ versus SR-$BI^{-/-}$ mice. All mice were maintained on a chow diet. Liver directed gene transfer of apoA-I was performed in apoA-$I^{-/-}$ mice by intravenous administration of control or apoA-I-containing adenovirus ($1X10^{11}$ particles per mouse) (provided by Dr. D. Rader, University of Pennsylvania), and apoA-I and HDL levels were measured 5 d after injection (Tsukamoto et al., 1997) (performed by the lab of Dr. D. Rader, University of Pennsylvania). In selected studies, male C57BL/6 mice were injected with the apoA-I-containing adenovirus and reendothelialization was evaluated. Five days following injury, animals were injected with 5% Evan's blue dye (Sigma), arteries were harvested, and the area of denudation (which incorporates the dye) was quantified in a blinded manner by image analysis using Scion Image (free software from NIH). On the day of injury there was similar initial denudation between comparison groups. All animal experiments were

approved by the Institutional Animal Care and Research Advisory Committee at UT Southwestern.

Isolation of HDL from SR-BI$^{+/+}$ or SR-BI$^{-/-}$ mice: The HDL fraction from SR-BI$^{+/+}$ or SR-BI$^{-/-}$ mice was isolated according to procedures described previously (Kitchens et al., 1999). Briefly, native HDL was isolated from plasma by sequential ultracentrifugal flotation in potassium bromide at a density of 1.21 g/ml. The lipoproteins were concentrated by recentrifugation and dialyzed thoroughly against cold 0.9% NaCl containing 0.25 mM EDTA (pH 8). The protein concentration of the HDL fraction was evaluated after dialysis.

Endothelial cell migration and lamellipodia formation in response to HDL from SR-BI$^{+/+}$ or SR-BI$^{-/-}$ mice: BAEC migration was evaluated as mentioned in the methods section of Chapter Two. BAEC were placed in DMEM + 1% LPDS for 16 h before the addition of 20-50 μg/ml HDL from SR-BI$^{+/+}$ or SR-BI$^{-/-}$ mice for 24 h and migration was evaluated. Lamellipodia formation was evaluated as mentioned in the methods section of Chapter Two. BAEC were placed in DMEM for 16 h before the addition of 50 μg/ml HDL from SR-BI$^{+/+}$ or SR-BI$^{-/-}$ mice for 5 min and lamellipodia formation was evaluated.

Immunohistochemistry: Carotid arteries of 10-15 week old male C57BL/6 mice were subjected to perivascular electric injury. Arteries of uninjured, 1 day-injured and 8-day injured mice were harvested and sectioned into 7 μm-thick sections. In selected studies, arteries of male apoA-$I^{+/+}$, apoA-$I^{-/-}$, SR-$BI^{+/+}$ or SR-$BI^{-/-}$ mice were harvested 5 days after injury. Sections were stained with 16.6 μg/ml anti-VWF antibody (Abcam), 2 μg/ml biotinylated-secondary antibody, 2.5 μg/ml HRP-streptavidin (Kirkegaard and Perry Laboratories), and diaminobenzidine (Sigma), and viewed under a microscope at 400X magnification.

Intimal Hyperplasia: Carotid arteries of apoA-$I^{+/+}$, apoA-$I^{-/-}$, SR-$BI^{+/+}$ or SR-$BI^{-/-}$ mice were subjected to perivascular electric injury. Four weeks after injury, arteries were harvested and sectioned into 7 μm-thick sections. Sections were stained with hematoxylin and eosin and viewed under light microscopy at 200X magnification to assess intimal and medial hyperplasia.

Blood Pressure by Radiotelemetry: SRBI knockout mice and their wild-type littermates (30-35 week-old SR-$BI^{+/+}$ and SR-$BI^{-/-}$ mice) were anesthetized with isoflurane at 2-3 % in a stream of 100% oxygen mixed with room air. A Data Sciences transmitter catheter (PAC20) was inserted into the left carotid artery via a 10 mm incision on the ventral neck region over the trachea, and the transmitter body was routed to a subcutaneous pocket in the left flank region. The incision

was closed with sterile, 6-0 Ethicon Opthalmic suture. Following recovery, mice were housed in individual cages in the animal facilities with a 12:12-hour dark-light cycle and provided with 6% fat diet and water ad libitum. Twenty-four-hour recordings were performed 7 days later when mice had regained their circadian heart rate and blood pressure rhythm. Blood pressure and heart rate data were collected and averaged during the period of monitoring of 1 week.

Results

Model of endothelial injury and reendothelialization in vivo

To determine if the mechanisms revealed in the *in vitro* studies of HDL-induced endothelial cell migration are operative *in vivo*, carotid artery reendothelialization studies were performed in mice. According to the procedures used by Brouchet et al., we subjected the left common carotid artery of mice to perivascular electric injury to cause denudation, and the contralateral artery served as the non-denuded control (sham) (Brouchet et al., 2001). The area of remaining denudation was determined postinjury by the injection of Evan's blue dye, which is incorporated in the region of denudation (**Figure 4-1**). In control C57BL/6 mice reendothelialization was complete by 7 d as indicated by minimal Evan's blue dye staining. Denudation of the endothelium after injury was confirmed by staining for von Willebrand factor (vWF), an endothelial-specific marker, 1 day after injury. Reendothelialization at day 8 post injury was also confirmed by vWF staining (**Figure 4-2**). No endothelial staining was seen in the no-Ab control which excluded the primary anti-vWF antibody from the staining procedure, thus confirming the specificity of the antibody.

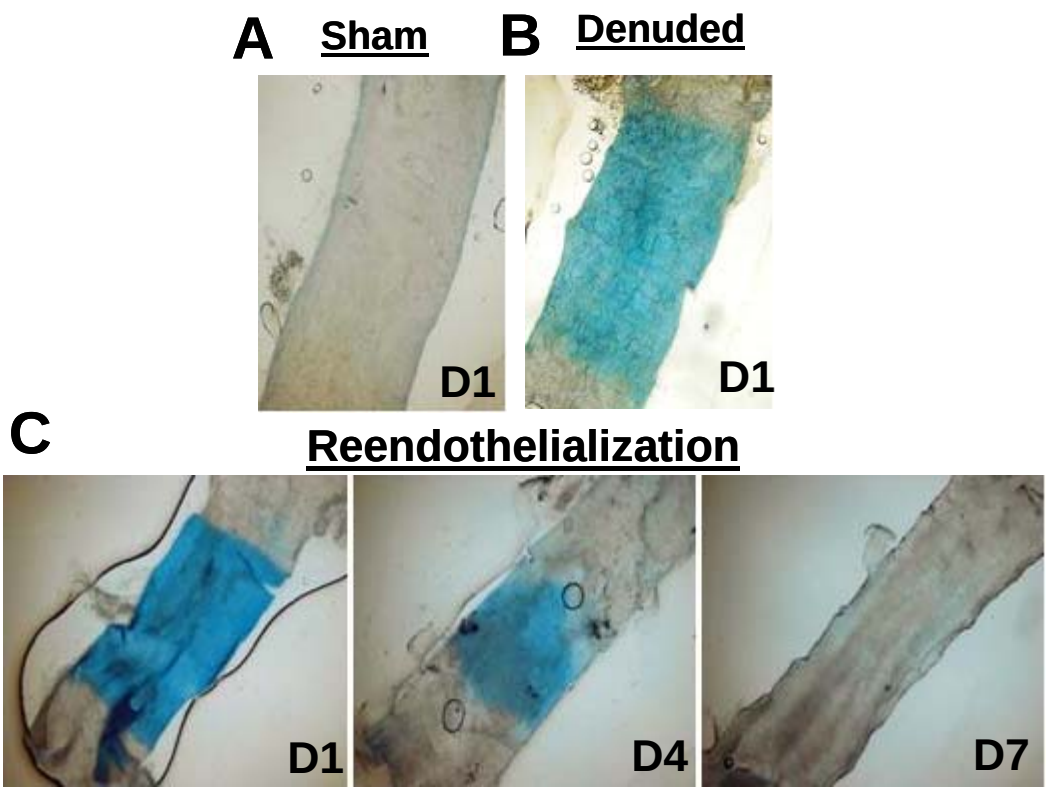

Figure 4-1: Mouse model of reendothelialization. The carotid arteries of C57BL/6 mice were subjected to sham procedure or perivascular electric injury to induce endothelial denudation. The area of denudation was evaluated using Evan's blue dye. The intimal surfaces of carotid arteries subjected to sham procedure and denudation are shown on d 1 in A and B, respectively. Representative images of the area of remaining denudation on d 1, 4 and 7 postinjury are shown in C.

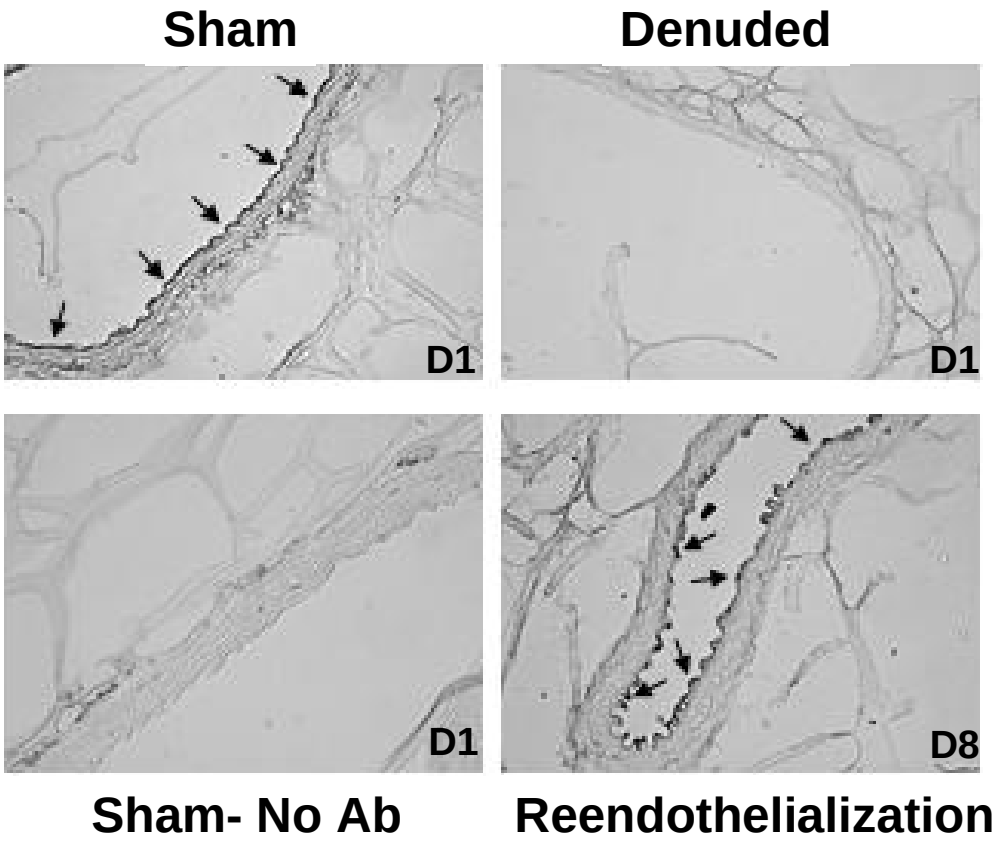

Figure 4-2: Denudation and recovery of endothelium after injury. The carotid arteries of C57BL/6 mice were subjected to perivascular electric injury to induce endothelial denudation. Arteries were sectioned into 7 μm sections and stained with anti-von Willebrand Factor antibody. Sham: uninjured artery; Denuded:1 d injured artery; Sham-No Ab: uninjured artery; Reendothelialization: 8 day injured artery. Arrows indicate vWF staining.

HDL and reendothelialization in vivo

To determine the role of HDL in reendothelialization *in vivo*, reendothelialization was compared 5 d postinjury in wild-type C57BL/6 mice (apoA-$I^{+/+}$) and in apoA-I null C57BL/6 mice (apoA-$I^{-/-}$), which have HDL cholesterol levels that are decreased by 83% compared to those of wild type mice (Williamson et al., 1992). Greater reendothelialization occurred in apoA-$I^{+/+}$ (**Figure 4-3A**) versus apoA-$I^{-/-}$ (**Figure 4-3B**) as indicated by the larger area of remaining denudation in apoA-$I^{-/-}$. Cumulative studies revealed that the area of remaining denudation was 52% larger in apoA-$I^{-/-}$ versus apoA-$I^{+/+}$ (**Figure 4-3C**). To confirm that the Evan's blue staining corresponds to areas of denudation, we subjected apoA-$I^{+/+}$ and apoA-$I^{-/-}$ mice to electric injury and 5 d later harvested the arteries, sectioned the arteries into 7 μm sections and stained the sections with the anti-vWF antibody. Results indicated that arteries from apoA-$I^{+/+}$ mice contained a high density of endothelial cells in the region of prior injury. However, endothelial density was much lower in the region of prior injury in the arteries from apoA-$I^{-/-}$ mice (**Figure 4-4**).

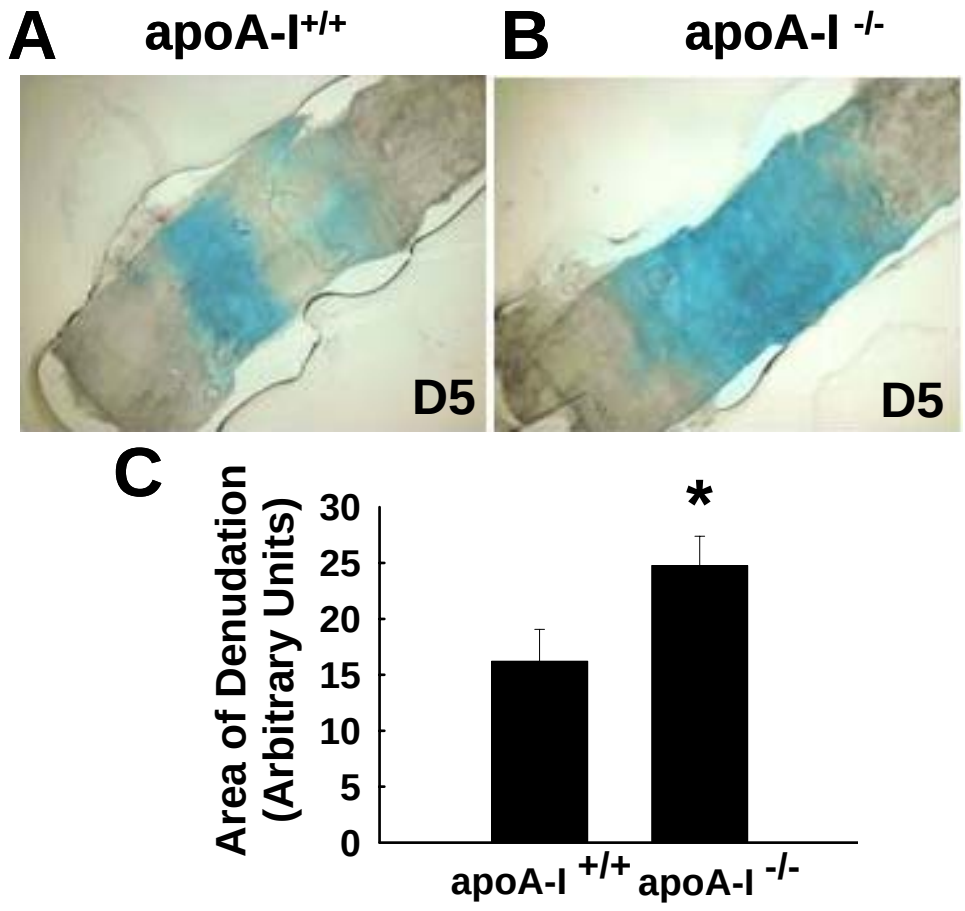

Figure 4-3: ApoA-I and HDL promote reendothelialization. apoA-I$^{+/+}$ or apoA-I$^{-/-}$ mice were subjected to perivascular electric injury of the carotid artery. Five d later the area of remaining denudation was evaluated using Evan's blue dye. The intimal surface of previously injured arteries from an apoA-I$^{+/+}$ and an apoA-I$^{-/-}$ mouse on d 5 are shown in **A** and **B**, respectively. (**C**) Area of denudation was quantified. Values are mean±SEM, *n*=11-14 mice/group**.** *, *P*<0.05 versus apoA-I$^{+/+}$.

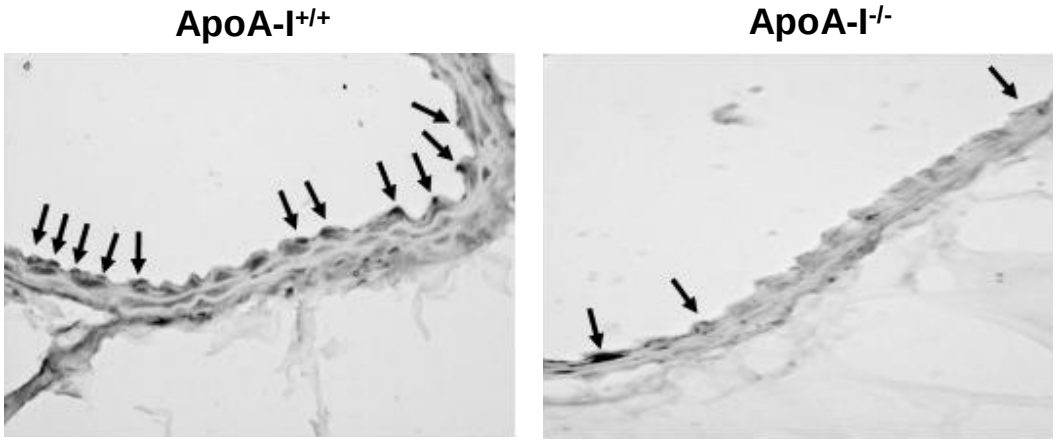

Figure 4-4: ApoA-I and HDL promote reendothelialization. apoA-$I^{+/+}$ or apoA-$I^{-/-}$ mice were subjected to perivascular electric injury of the carotid artery. Five d later the area of remaining denudation was evaluated by staining with anti-vWF antibody. The arterial sections of previously injured arteries from an apoA-$I^{+/+}$ and an apoA-$I^{-/-}$ mouse on d 5 are shown in A and B, respectively. Arrows indicate vWF staining.

To test if normal reendothelialization can be rescued by reconstitution of apoA-I expression in apoA-$I^{-/-}$ mice, liver-directed gene transfer of human apoA-I was performed at the time of artery injury. The predicted increase in plasma apoA-I and HDL was confirmed by plasma lipoprotein analysis at the time of study termination 5 d postinjection. ApoA-$I^{-/-}$ mice injected with control adenovirus had HDL levels of 19.6±7.7 mg/dl. In contrast, apoA-$I^{-/-}$ mice receiving apoA-I-containing adenovirus had apoA-I levels of 148.4±49.4 mg/dl ($p<0.05$ vs control) and HDL levels of 69.7±25.2 mg/dl ($p<0.05$ vs control). Evan's blue dye incorporation 5 d after injury demonstrated that in comparison with apoA-$I^{-/-}$ mice given control adenovirus (**Figure 4-5A**), mice injected with

apoA-I-containing adenovirus displayed greater reendothelialization (**Figure 4-5B**). Quantitation in multiple mice indicated that there was 40% less denudation following the reconstitution of apoA-I expression in apoA-I$^{-/-}$ (**Figure 4-5C**). These cumulative observations indicate that apoA-I and HDL are important modulators of reendothelialization *in vivo*.

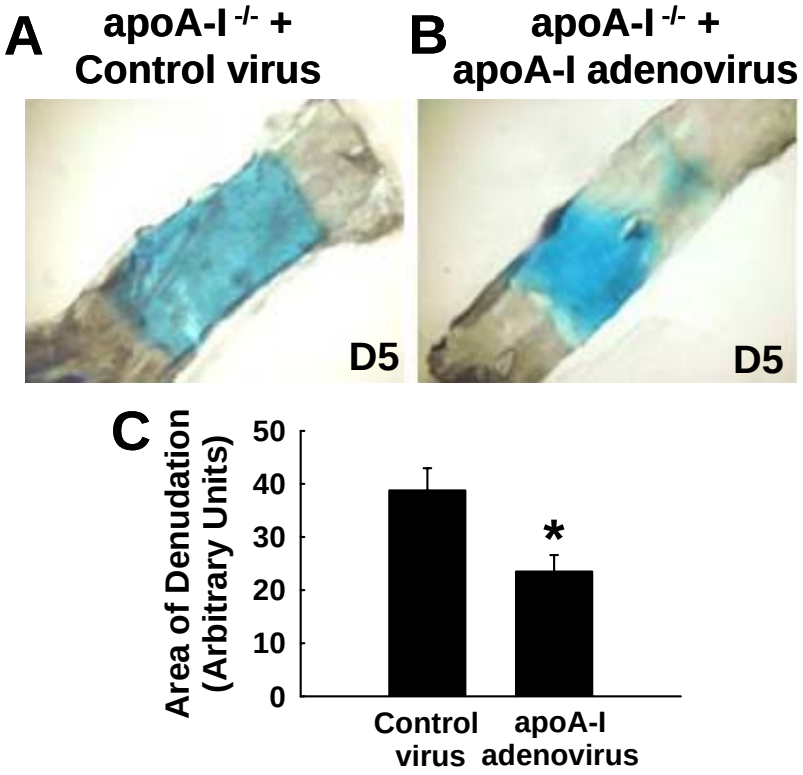

Figure 4-5: Reconstitution of apoA-I expression in apoA-I$^{-/-}$ mice normalizes reendothelialization. apoA-I$^{-/-}$ mice received control adenovirus or apoA-I containing adenovirus on the day of injury and the area of remaining denudation was evaluated 5 d later (**A** and **B**, respectively). (**C**) Area of denudation was quantified. Values are mean±SEM n=7 mice/group. *, P<0.05 versus control virus.

To determine the effect of increased apoA-I and HDL in wild type mice, human apoA-I-containing adenovirus was administered to wild-type C57BL/6 mice. Liver-directed gene transfer of the adenovirus effectively raised apoA-I and HDL cholesterol levels (control virus: HDL=50.6+/-3.8 mg/dl; apoA-I adenovirus: apoA-I= 372.6+/-91.3 and HDL= 174.2+/-32.3), but did not alter reendothelialization compared with control adenovirus (15.7±6.0 vs 15.7±5.1 arbitrary units for area of remaining denudation, n=8 and n=7, respectively).

SR-BI and reendothelialization in vivo

Having demonstrated that there is a major contribution of apoA-I/HDL to reendothelialization *in vivo*, we next determined the role of SR-BI in studies of SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice. The area of remaining denudation was evaluated 5 d after thermal artery injury in 12-16 week old male mice. Greater reendothelialization occurred in SR-BI$^{+/+}$ (**Figure 4-6A**) versus SR-BI$^{-/-}$ mice (**Figure 4-6B**) as indicated by the larger area of Evan's blue dye incorporation in the latter. Cumulative studies indicated that the area of remaining denudation was 44% larger in SR-BI$^{-/-}$ versus SR-BI$^{+/+}$ (**Figure 4-6C**). Comparable findings were obtained in older mice studied at 30-35 weeks of age (data not shown).

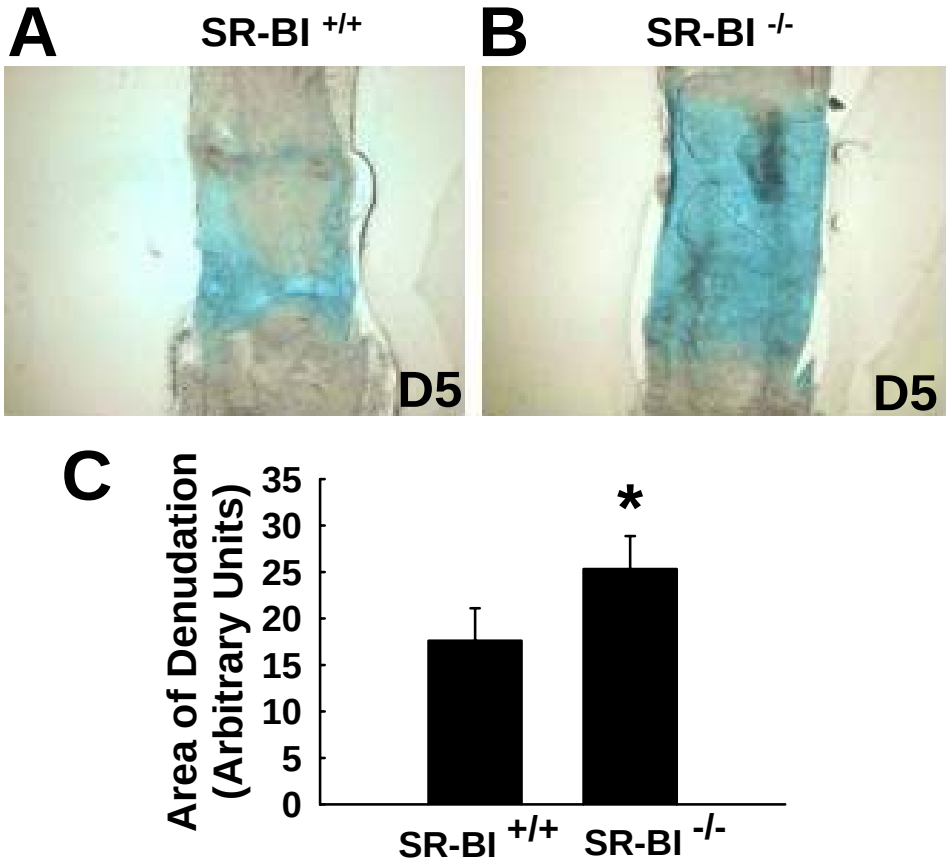

Figure 4-6: SR-BI promotes reendothelialization. SR-BI$^{+/+}$ or SR-BI$^{-/-}$ mice were subjected to perivascular electric injury of the carotid artery. Five days later the area of remaining denudation was evaluated using Evan's blue dye. The intimal surface of previously injured arteries from an SR-BI$^{+/+}$ and an SR-BI$^{-/-}$ mouse on d 5 are shown in **A** and **B**, respectively. (**C**) Area of denudation was quantified. Values are mean±SEM, *n*=7-8 mice/group. *, *P*=0.05 versus SR-BI$^{+/+}$.

To confirm that the Evan's blue staining corresponds to areas of denudation, we subjected SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice to electric injury and 5d later harvested the arteries, sectioned the arteries into 7 µm sections and stained

the sections with the anti-vWF antibody. Results indicated that arteries from SR-BI$^{+/+}$ mice contained a high density of endothelial cells in the region of prior injury. However, endothelial density was much lower in the region of prior injury in the arteries from SR-BI$^{-/-}$ mice (**Figure 4-7**). Thus, the phenotype of attenuated reendothelialization observed with lowered apoA-I and HDL levels was recapitulated by the loss of SR-BI, thereby providing mechanistic linkage of apoA-I, HDL and SR-BI in the modulation of reendothelialization *in vivo*.

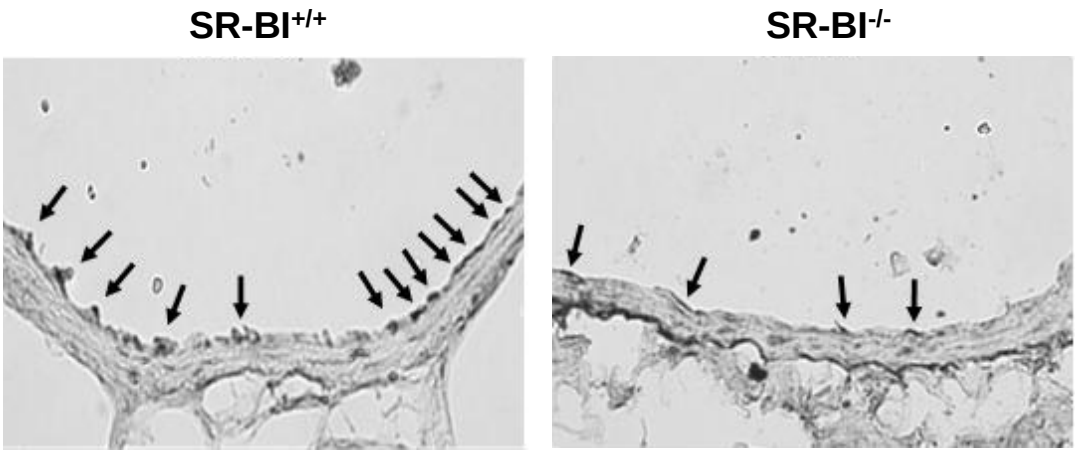

Figure 4-7: SR-BI promotes reendothelialization. SR-BI$^{+/+}$ or SR-BI$^{-/-}$ mice were subjected to perivascular electric injury of the carotid artery. Five d later the area of remaining denudation was evaluated by staining with anti-vWF antibody. The arterial sections of previously injured arteries from an SR-BI$^{+/+}$ and an SR-BI$^{-/-}$ mouse on d 5 are shown in A and B, respectively.

Our prior work has shown that HDL and SR-BI have potent promodulatory effects on eNOS (Uittenbogaard et al., 2000; Yuhanna et al., 2001). Since diminished eNOS function causes hypertension (Shaul, 2002) and hypertension is associated with endothelial injury (Limas et al., 1980; Strawn et al., 1997), we determined if the blunted reendothelialization in SR-BI$^{-/-}$ mice is related to hypertension. Using radiotelemetry, we performed chronic blood pressure (BP) measurements in SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice at 30-35 weeks of age. Systolic, diastolic and mean BP as well as heart rate were similar in SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (**Table 4-1**). Therefore, the attenuation of reendothelialization in SR-BI$^{-/-}$ mice is not due to hypertension.

	SR-BI$^{+/+}$ (n=6)	SR-BI$^{-/-}$ (n=7)
Systolic BP (mm Hg)	124 ± 5	128 ± 3
Diastolic BP (mm Hg)	93 ± 2	94 ± 3
Mean BP (mm Hg)	108 ± 3	106 ± 2
Heart Rate (beats/min)	616 ± 26	593 ± 21

Table 4-1: Blood pressure and heart rate are similar in SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice. Values are mean±SEM.

Since SR-BI$^{-/-}$ mice have heterogeneous, enlarged HDL particles with increased cholesterol content compared to those of SR-BI$^{+/+}$ mice (Rigotti et al., 1997), it is possible that the differences in reendothelialization observed in SR-BI$^{+/+}$ and SR-BI$^{-/-}$ are due to the disparities in the HDL particle. To address this possibility, the capacity to activate endothelial cell migration was compared *in vitro* using HDL isolated from SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice. Endothelial cells were wounded and treated with human HDL (positive control, 50 μg/ml) or SR-BI$^{+/+}$ or SR-BI$^{-/-}$ HDL (20 or 50 μg/ml) for 24 h. Endothelial cell migration stimulated by HDL from SR-BI$^{-/-}$ mice was at least as robust as that observed with HDL from SR-BI$^{+/+}$ mice (**Figure 4-8A**). In parallel studies of lamellipodia formation induced by HDL (50 μg/ml for 5 min), responses were identical for HDL from SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (**Figure 4-8B**). These observations indicate that the features of HDL required to activate endothelial cell migration are not altered in SR-BI$^{-/-}$ mice. Therefore it is not differences in the "ligand" for SR-BI which underlie the attenuation in reendothelialization in SR-BI$^{-/-}$ mice, and the evidence for a major role for SR-BI is further strengthened.

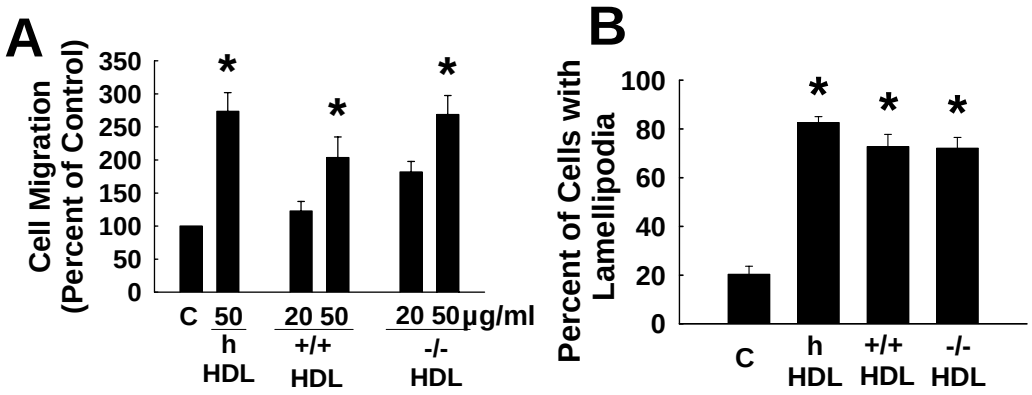

Figure 4-8: HDL from SR-BI$^{-/-}$ mice is as potent as HDL from SR-BI$^{+/+}$ mice in promoting endothelial cell migration and lamellipodia formation. (A) BAEC grown to near-confluence were wounded and treated with media alone (control, C), or media plus 50 µg/ml human (h) HDL or 20 or 50 µg/ml HDL from SR-BI$^{+/+}$ or SR-BI$^{-/-}$ mice for 24 h, and migration was evaluated. Values are mean±SEM, *n*=4. *, *P*<0.05 versus control. **(**B) BAEC were treated with media alone (control, C), or media plus 50 µg/ml human (h) HDL or 50 µg/ml HDL from SR-BI$^{+/+}$ or SR-BI$^{-/-}$ mice for 5 min, and the percent of cells with lamellipodia was quantified using Alexa-568-phalloidin. Values are mean±SEM, *n*=3. *, *P*<0.001 versus control.

HDL and SR-BI and neointima formation after injury

Disruption of the endothelial layer can lead to an increase in neointima formation in hypercholesterolemic mice (De Geest et al., 1997). In order to determine if apoA-I$^{-/-}$ and SR-BI$^{-/-}$ mice subjected to electric injury are more susceptible to neointima formation compared to their respective wild type controls, the carotid arteries of apoA-I$^{+/+}$, apoA-I$^{-/-}$, SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice were subjected to electric injury, harvested after 4 weeks, sectioned into 7 µm sections, and stained with hematoxylin and eosin. The thicknesses of the intimal

and medial layers of the arteries from apoA-I$^{-/-}$ mice were similar to those of wild type mice, and no neointimal formation was noted (**Figure 4-9**). Similar results were observed in the SR-BI$^{-/-}$ mice compared to their wild type littermates (**Figure 4-10**).

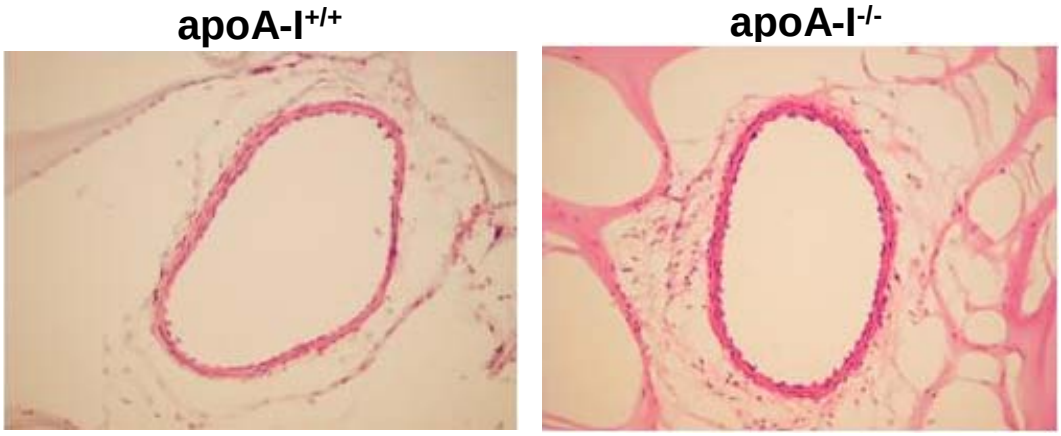

Figure 4-9: Initimal and medial thickening is absent in apoA-I$^{-/-}$ mice after thermal injury. Carotid arteries of apoA-I$^{+/+}$ and apoA-I$^{-/-}$ mice were subjected to perivascular electric injury, and 4 weeks later they were sectioned and stained with hematoxylin and eosin. n= 4 mice/group.

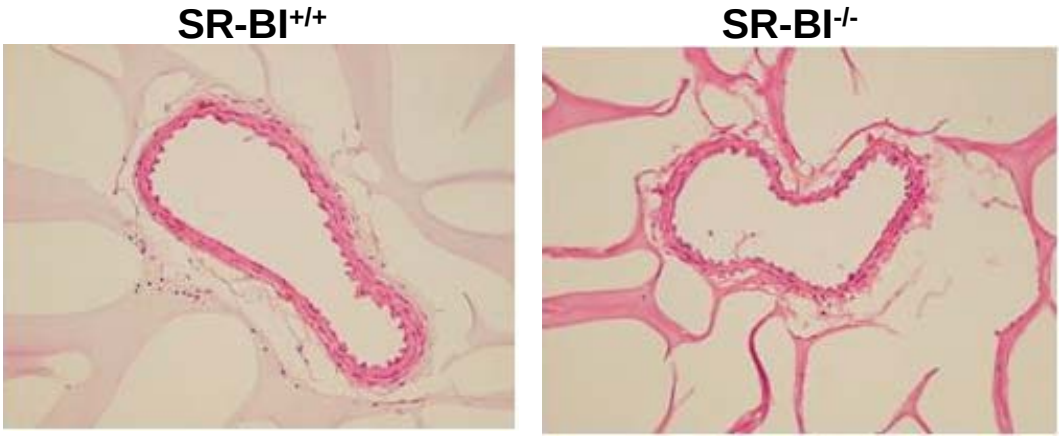

Figure 4-10: Initimal and medial thickening is absent in SR-BI$^{-/-}$ mice after thermal injury. Carotid arteries of SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice were subjected to perivascular electric injury, and 4 weeks later they were sectioned and stained with hematoxylin and eosin. n= 4 mice/group.

Conclusions

In mouse models of atherosclerosis, apoA-I and SR-BI provide atheroprotection (Kozarsky et al., 2000; Paszty et al., 1994; Plump et al., 1994; Ueda et al., 2000). The observation of HDL-mediated endothelial cell migration *in vitro* indicates that HDL may provide atheroprotection by preserving the integrity of the endothelial layer in addition to stimulating reverse cholesterol transport and NO production. In order to test the role of HDL and SR-BI in endothelial repair *in vivo*, we evaluated the extent of reendothelialization in carotid arteries of apoA-$I^{-/-}$ and SR-$BI^{-/-}$ mice compared to wild type mice.

The extent of reendothelialization in the apoA-$I^{+/+}$ mice was much greater than that of apoA-$I^{-/-}$ mice as evaluated by Evan's blue dye staining. We also confirmed the decreased endothelial cell recovery in the apoA-$I^{-/-}$ mice compared to apoA-$I^{+/+}$ mice in the region of prior injury by immunostaining for an endothelial cell marker. These results in addition to the rescue of reendothelialization with apoA-I adenovirus injection in apoA-$I^{-/-}$ mice indicate that apoA-I and HDL promote reendothelialization. Wild type C57BL/6 mice injected with the apoA-I-containing adenovirus did not show increased reendothelialization compared to wild type mice given empty virus. These cumulative observations indicate that apoA-I and HDL at or below normal levels promote endothelial monolayer integrity *in vivo*.

SR-BI$^{-/-}$ mice displayed attenuated reendothelialization compared to SR-BI$^{+/+}$ mice after thermal injury. We also observed an increased endothelial cell recovery in the SR-BI$^{+/+}$ mice compared to SR-BI$^{-/-}$ mice in the region of prior injury by vWF staining, thereby confirming the results of the Evan's blue staining. These results indicate that SR-BI is an important modulator of reendothelialization.

The studies of neointima formation in the apoA-I$^{-/-}$ and SR-BI$^{-/-}$ mice compared to their respective wild type controls showed no increase in intimal thickening in the null mice after injury, and medial thickness also did not change. These results are perhaps not surprising because prior studies using guidewire-induced injury of the endothelium did not find neointima formation in the carotid artery under conditions of normocholesterolemia (C57BL/6) but did so in the presence of overt hypercholesterolemia (apoE$^{-/-}$ mice) (De Geest et al., 1997). Although some studies have reported neointima formation in C57BL/6 after guidewire injury or thermal injury in the femoral artery (Carmeliet et al., 1997; Roque et al., 2000), the physiology of the femoral artery differs from that of the carotid artery. Further studies are required to determine if apoA-I$^{-/-}$ and SR-BI$^{-/-}$ mice fed a high cholesterol diet or crossed to apoE$^{-/-}$ mice display increased neointima formation after thermal injury compared to their controls.

CHAPTER FIVE: Overall Conclusions and Current Questions

Circulating levels of HDL cholesterol and the major HDL apolipoprotein apoA-I are inversely related to the risk for cardiovascular disease (Gordon and Rifkind, 1989). In addition, interventional clinical trials indicate that modest increases in HDL independently result in a significant reduction in overall cardiovascular events (Taylor et al., 2004). Thus, HDL is not simply a marker of decreased vascular disease risk but an important mediator of vascular health. Although RCT has been considered to be the primary mechanism for HDL-related cardiovascular protection, recent human and animal studies have suggested that the atheroprotective nature of HDL is not sufficiently explained by RCT (Groen et al., 2001; Jolley et al., 1998; Osono et al., 1996). As such, the basis for the protective nature of HDL/apoA-I remains poorly understood.

HDL stimulates NO production in endothelial cells by binding to SR-BI and stimulating a kinase cascade leading to the activation of eNOS (Mineo et al., 2003; Yuhanna et al., 2001). During the initiating phase of numerous vascular diseases, the earliest threat to the vascular wall is from exposure to cytokines, disturbed shear stress, reactive oxygen species, and mechanical denudation. Efficient repair of the endothelium is imperative in preserving the endothelial barrier and preventing endothelial dysfunction. In these studies we show that HDL and SR-BI stimulate endothelial cell migration after wounding *in vitro* with

potency equivalent to the well-known angiogenic factor VEGF. We further demonstrate that phospholipid, apoA-I and cholesterol components of HDL are sufficient to initiate this cellular response. Although the mechanism by which this occurs is unknown, the HDL/SR-BI ligand-receptor pair promote selective cholesterol flux and this characteristic may account for the increased migration observed when cholesterol is added in the reconstituted HDL particles. As observed by Ghosh et al., the addition of cholesterol to endothelial cells increases microviscosity of the plasma membrane and induces migration (Ghosh et al., 2002). This is seen after the addition of growth factors as well. A proposed mechanism for increased migration by increased microviscosity is the localization of Rac to plasma membrane indicative of its increased activity. The observations in the current study of increased migration after addition of cholesterol are consistent with the observations made by Ghosh et al. Although the mechanism by which the cholesterol component in HDL increases migration is unclear, our recent work suggests that SR-BI may act as a cholesterol sensor on the plasma membrane (Assanasen et al., 2005). In this investigation of the most proximal events in HDL signaling to the kinases regulating eNOS, we found that the process requires cholesterol flux, the C-terminal transmembrane domain of SR-BI which directly binds cell cholesterol, and the C-terminal PDZ-interacting domain of SR-BI. Further studies are required to determine how cholesterol flux mediated by HDL promotes endothelial cell migration.

The signaling cascade mediating HDL and SR-BI stimulation of endothelial cell migration is outlined in **Figure 5-1**. Prior studies of HDL activation of eNOS revealed that the lipoprotein activates Src family kinases which activate PI3 kinase, leading to Akt kinase and MAPK stimulation and greater eNOS enzymatic activity (Mineo et al., 2003). The current work demonstrates that the activation of these kinases by HDL-SR-BI is also mechanistically linked to the promotion of endothelial cell migration, and that this response is independent of effects on eNOS. This finding is interesting especially since HDL is a potent stimulator of NO production and eNOS has been implicated in endothelial cell migration and angiogenesis in response to various agonists (Ashton and Ware, 2004; Chen et al., 2004; Kawasaki et al., 2003; Shizukuda et al., 1999). In addition, we show that HDL causes rapid Rac activation in concert with lamellipodia formation in an SR-BI-dependent manner, and that Rac is required for both increased lamellipodia formation and ultimate cell migration. This is consistent with numerous studies which have demonstrated the requirement for Rac in migration and cytoskeletal rearrangement (Nobes and Hall, 1995; Nobes and Hall, 1999; Ridley et al., 1992; Ridley, 2001). In further studies we demonstrate that Src family kinases, PI3K and MAPK are all necessary for HDL-induced Rac activity whereas Rac knockdown does not alter kinase activation, indicating that the kinases reside upstream of Rac in the series

of events by which HDL and SR-BI regulate endothelial cell motility (Figure 5-1, solid arrows).

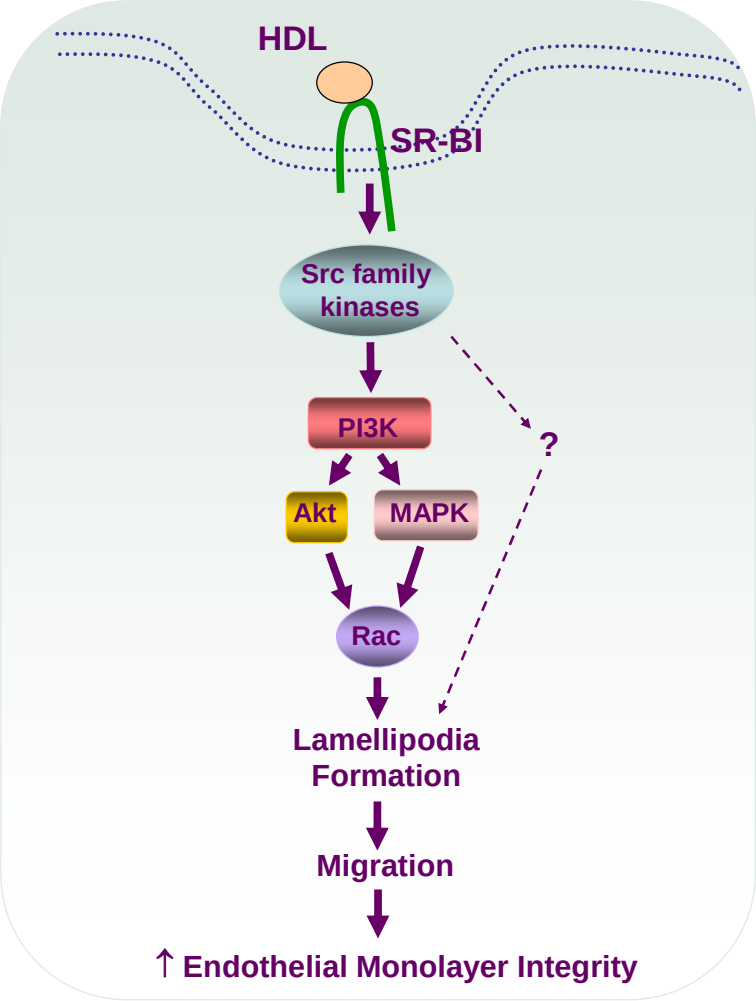

Figure 5-1: HDL signaling through SR-BI promotes endothelial cell migration and monolayer integrity. HDL binding to SR-BI sequentially activates Src family kinases, PI3K, and Akt kinase and MAPK. These events lead to the activation of Rac GTPase and lamellipodia formation, and ultimately to enhanced endothelial cell migration which promotes endothelial monolayer integrity (solid arrows). Alternative SR-BI- and Src family kinase-dependent processes may also mediate initial lamellipodia formation (dashed arrows).

It should be noted that although antagonism of PI3 kinase or MAPK activity prevented both HDL-induced Rac activation and cell migration, these interventions did not alter lamellipodia formation. As such, alternative SR-BI- and Src family kinase-dependent processes may also mediate initial lamellipodia formation (Figure 5-1, dashed arrows). In addition, HDL-related PI3K and MAPK activation may modify signaling events required for processes other than lamellipodia formation such as cell adhesion to matrix contacts, contraction, and release from matrix contacts which are also involved in cell migration. Collectively these studies of HDL activation of endothelial cell motility have revealed that multiple, sequential signaling events occur in endothelium in response to the lipoprotein. Whether comparable signaling mediates HDL actions in other cell types is yet to be determined.

In order to delineate the components of HDL and SR-BI responsible for mediating endothelial migration, it is important to reconstitute the HDL-SR-BI system in a cell type which does not contain SR-BI. When we attempted to test migration after expressing SR-BI in Cos 7, Cos M6, Chinese Hamster Ovary, HeLa and Human Embryonic Kidney 293 cells, these cell types did not migrate in response to HDL but migrated in response to serum. These results suggest that endothelial cells may contain certain signaling molecules or adaptor proteins which mediate HDL-induced endothelial cell migration which are absent in other cell types. Due to this result, it was challenging to study the structural features of

SR-BI. Further efforts are required to establish such a model system to investigate how SR-BI governs HDL-mediated endothelial cell migration.

In studies performed in mice, in the context of all other endogenous factors regulating endothelial cell phenotype *in vivo*, HDL/apoA-I and SR-BI promoted reendothelialization of carotid artery. The thermal injury model provides a quantitative measure of reendothelialization. Using this model, we show that apoA-I deficiency leads to decreased reendothelialization and that endothelial repair is accelerated when apoA-I is supplemented by liver-directed gene transfer in apoA-I$^{-/-}$ mice. An increase in apoA-I and HDL levels in wild type mice does not lead to accelerated reendothelialization, indicating that apoA-I and HDL at or below normal levels promote endothelial monolayer integrity *in vivo*. SR-BI deficiency also blunts reendothelialization in after injury. This represents an entirely novel role for the HDL/SR-BI tandem, complementing the capacity of the lipoprotein and the receptor to regulate cell cholesterol flux and endothelial NO production (Connelly and Williams, 2004; Drew et al., 2004; Krieger, 1999; Mineo et al., 2003; Uittenbogaard et al., 2000; Yuhanna et al., 2001). As mentioned earlier, the role of the adaptor protein PDZK1 has been suggested in HDL-mediated eNOS activation. In order to test the requirement for PDZK1 in vivo, reendothelialization in PDZK1 null mice should be tested. If PDZK1 is required, reendothelialization would be blunted in the null mice although the mice have adequate levels of HDL and SR-BI.

The present observations reveal a novel series of mechanisms by which apoA-I/HDL and SR-BI are positive modulators of endothelial cell motility. In addition, the physiological relevance of the findings *in vitro* are demonstrated *in vivo* where apoA-I/HDL and SR-BI mediate reendothelialization after injury. Collectively, these findings provide a new framework for understanding how HDL promotes vascular health.

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VITAE

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