High-Density Lipoprotein Promotes Endothelial Cell Migration and Reendothelialization via Scavenger Receptor-B Type I


Abstract—Vascular disease risk is inversely related to circulating levels of high-density lipoprotein (HDL) cholesterol. However, 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. Here, 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 after perivascular electric injury is blunted in apolipoprotein A-I−/− mice, and reconstitution of apolipoprotein A-I expression rescues normal reendothelialization. Furthermore, reendothelialization is impaired in SR-BI−/− mice. Thus, HDL stimulates endothelial cell migration via SR-BI-initiated signaling, and these mechanisms promote endothelial monolayer integrity in vivo. (Circ. Res. 2006;98:63-72.)

Key Words: high-density lipoprotein • endothelium • migration

The risk of atherosclerosis is inversely related to circulating levels of high-density lipoprotein (HDL) cholesterol.1,2 HDL classically functions in reverse cholesterol transport (RCT), removing cholesterol from peripheral tissues and delivering it to the liver and steroidogenic organs by binding of the HDL apolipoprotein apolipoprotein A-I (apoA-I) to the HDL receptor scavenger receptor B type I (SR-BI).3,4 In mouse models of atherosclerosis, both apoA-I and SR-BI provide atheroprotection.5,6 However, as animal studies show that RCT is not dictated by circulating levels of HDL or apoA-I,7–9 the changes in atherosclerosis risk related to varying HDL and apoA-I levels may not be explained by differences in RCT. In addition, clinical studies suggest that the risk for restenosis after vascular intervention may be inversely related to HDL levels,10,11 and the provision of apoA-I or HDL attenuates neointima formation after artery injury in hypercholesterolemic animal models.12,13 There is evidence that HDL also inhibits low-density lipoprotein oxidation and adhesion molecule expression in cultured endothelial cells, but the magnitude and impact of these processes in vivo is uncertain.14,15 As such, there is considerable evidence that HDL affords protection from vascular disease, but the mechanisms underlying the protection remain poorly understood.

Whereas it has been appreciated for some time that SR-BI is expressed in hepatocytes and steroidogenic tissues, we and others16–20 have more recently demonstrated that SR-BI is also expressed in endothelium, where it mediates promodulatory effects of HDL on endothelial nitric oxide synthase (eNOS) to increase the abundance of NO. In addition to generating NO, an intact endothelial cell monolayer modulates local hemostasis and thrombolysis and provides a nonpermeable barrier protecting smooth muscle cells from circulating growth-promoting factors. Disruptions of endothelial cell monolayer integrity, either by gross denudation related to a vascular intervention or gap formation between cells due to disturbed shear stress, place the arterial wall at greater risk for vascular disease.21–23 Furthermore, whereas repeated endothelial removal worsens vascular lesion severity,24 enhanced reendothelialization blunts lesion formation.25,26 To better understand the basis of HDL-related vascular protection, we designed experiments to determine

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whether HDL functioning through SR-BI promotes endothelial cell migration and the maintenance of endothelial monolayer integrity. HDL-induced migration, lamellipodia formation, and Rac GTPase activation were interrogated in cultured endothelium. Because HDL activation of eNOS is mediated by Src kinases, phosphatidylinositol 3-kinase (PI3K)/Akt kinase and p44/42 mitogen-activated protein kinase (MAPK), the roles of these signaling cascades in HDL modulation of Rac GTPase and migration were determined. Because NO is critically involved in endothelial migration and angiogenesis, dependence on NO was also evaluated. Furthermore, the impact of loss of SR-BI was investigated. Moreover, to determine whether these processes are operative in vivo, carotid artery reendothelialization was assessed after perivascular electric injury in mice in which apoA-I/HDL/SR-BI status was genetically manipulated.

Materials and Methods

Additional details are provided in the online-only data supplement, available at http://circres.ahajournals.org.

Cell Culture

Bovine aortic endothelial cells (BAECs) were harvested using procedures reported previously with minor modifications, cultured in endothelial growth medium-2 (Cambrex Bioscience) with 5% fetal bovine serum (Sigma), and studied at passages 5 to 9.

Endothelial Cell Migration Assay

BAECs were grown to near confluence, and a defined region of cells was removed with a razor blade. Cells were treated for 24 hours and then fixed, and the number of cells that had migrated past the wound edge was quantified.

Cytoskeletal Changes in Endothelial Cells

BAECs were plated onto glass coverslips, treated for 0 to 30 minutes, fixed and stained with Alexa 568-phalloidin (Molecular Probes, Inc), and viewed under a fluorescent microscope. The percent of cells with lamellipodia was quantified.

Rac Activity Assay

After treatment, Rac activity in BAECs was measured as previously described with minor modifications.

Small Interfering RNA for Rac and SR-BI

Double-stranded RNA (dsRNA) sequences directed at bovine Rac or SR-BI were transfected into cells, and expression of the proteins and functional and signaling readouts were determined 24 to 48 hours later.

Carotid Artery Reendothelialization

Carotid artery reendothelialization was studied after perivascular electric injury in mice by assessing Evan’s blue dye uptake. Endothelial denudation and recovery after injury was confirmed by immunohistochemistry for von Willebrand factor (vWF). Study groups included wild-type mice, apoA-I/+/+ or SR-BI/−/− mice, and apoA-I/−/+ mice with adenoviral reconstitution of apoA-I expression.

Blood Pressure by Radiotelemetry

Chronic blood pressure measurements were performed in 30- to 35-week-old SR-BI/+ and SR-BI−/− mice by radiotelemetry.

Statistical Analysis

All data are presented as mean±SEM. ANOVA with Neuman-Keuls post hoc testing was used to assess differences between 3 or more groups. Differences in reendothelialization were evaluated by Mann-Whitney tests. Significance was set at P<0.05.

Results

HDL and Endothelial Cell Migration

To determine the effect of HDL on endothelial cell migration, BAECs were wounded and treated with HDL. The lipoprotein caused a marked increase in migration (Figure 1A), with 2.8-, 3.6-, and 4-fold increases noted with 20, 50, and 100 μg/mL HDL, respectively (Figure 1B), and the response to HDL was comparable to the response to vascular endothelial growth factor (VEGF) (Figure 1C). It has been previously reported that HDL activates endothelial cell migration and that this may be dependent on cargo molecules such as sphingosine-1-phosphate (S-1-P), which induces migration in a pertussis toxin (Ptx)-sensitive manner. To determine the contribution of S-1-P in HDL-induced migration, responses to HDL or S-1-P were assessed in the absence or presence of Ptx. Whereas S-1-P-induced migration was prevented by Ptx, HDL-mediated migration was not affected (Figure 1D). To determine whether the apolipoprotein, phospholipid, and cholesterol components of HDL are sufficient to stimulate migration, BAECs were treated with lipoprotein (Lp)2A-I particles reconstituted with 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), cholesterol, and lipid-free recombinant apoA-I. Lp2A-I containing POPC, cholesterol, and apoA-I at molar ratios of 80:5:1 caused migration comparable to that promoted by native HDL (Figure 1E). These observations indicate that HDL stimulates endothelial cell migration and that the phospholipid, cholesterol, and apoA-I components are sufficient to mediate the process.

HDL, Lamellipodia Formation, and Rac GTPase

Because cell migration begins with key changes in the actin cytoskeleton, including the formation of lamellipodia, we tested the initial effects of HDL on the cytoskeleton. Under control conditions, BAEC displayed stress fibers and few lamellipodia (Figure 2A). Within 1 minute of HDL exposure, there was a decrease in stress fibers, an increase in lamellipodia, and membrane ruffling. This effect became more apparent at 5 minutes, the number of lamellipodia decreased at 15 minutes, and cells appeared similar to control at 30 minutes. As Rac GTPase mediates lamellipodia formation, we determined whether Rac is activated by HDL. Paralleling the findings for lamellipodia formation, there was an increase in Rac activity with HDL within 1 minute (Figure 2B). Activity was maximal at 5 minutes and decreased thereafter.

Role of Kinase Activation

To further delineate the mechanisms by which HDL stimulates endothelial cell migration, we determined whether the...
process entails the kinases that have been implicated in HDL-mediated eNOS activation.17 Whereas the Src kinase inhibitor PP2 did not affect basal migration, HDL-stimulated migration was blunted by 72% by PP2 (Figure 3A, left panel). PP3, the negative control compound for PP2, had no effect (data not shown). Treatment with the PI3K inhibitor LY294002 did not affect basal migration, but HDL-stimulated migration was fully inhibited (Figure 3A, middle panel). PD 98059, which inhibits the MAPK pathway, did not alter basal migration, but HDL-induced migration was attenuated by 84% (Figure 3A, right panel). Therefore, HDL-induced endothelial cell migration is dependent on Src family kinases, PI3K, and MAPK.

The roles of Src kinases, PI3K, and MAPK in HDL-induced lamellipodia formation were also evaluated. BAEC pretreatment with PP2 had no effect on the cytoskeleton at baseline. However, the 14-fold increase in lamellipodia formation with HDL (Figure 3B, left panel) was blunted by 87% by PP2. PP3 had no effect (data not shown). In contrast, both LY294002 and PD98059 did not modify lamellipodia formation with HDL (Figure 3B, middle and right panels, respectively). The efficacy of LY294002 and PD98059 was confirmed in studies of HDL-stimulated Akt and MAPK phosphorylation (data not shown).

The role of kinases in Rac activation by HDL was also investigated. Whereas treatment with PP2 did not alter basal activity, HDL-induced Rac activation was blunted by 67% (Figure 3C, left panel). Treatment with LY294002 did not affect basal Rac activity but it attenuated HDL-mediated Rac activation by 86% (Figure 3C, middle panel). Basal Rac activity was not affected by PD98059, but HDL-induced Rac activation was diminished by 82% (Figure 3C, right panel). Therefore, Src family kinases, PI3K, and MAPK are required for HDL-induced Rac activity. To verify the sequence of kinase and Rac activation, Rac was knocked-down with siRNA and HDL-induced kinase phosphorylation was tested. HDL caused comparable phosphorylation of Src, Akt, and MAPK in cells transfected with control and Rac siRNA (Figure 3D). Thus, Rac resides downstream of the kinases in the signaling cascade by which HDL activates endothelial cell migration.

Role of eNOS
Because HDL stimulates eNOS19 and NO promotes endothelial cell migration and angiogenesis,27 we determined whether eNOS is required for HDL-mediated migration. The treatment of BAECs with the NOS antagonist Nω-Nitro-L-arginine methyl ester (L-NAME) did not affect basal migration, and HDL-induced endothelial cell migration was also not attenuated (Figure 4A). In contrast, VEGF-stimulated migration was blocked by L-NAME (Figure 4B). L-NAME did not alter the basal number of cells displaying lamellipodia, and lamellipodia formation in response to HDL was not changed (Figure 4C). The efficacy of L-NAME was verified by confirming 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,38 the promotion of endothelial cell migration by HDL is NO-independent.

Role of SR-BI
To determine whether SR-BI plays a role in HDL-mediated endothelial cell migration, SR-BI was knocked down by...
siRNA (Figure 5A). Whereas cells transfected with control siRNA displayed an 8.5-fold increase in migration with HDL (Figure 5B), cells transfected with SR-BI siRNA did not. In parallel, cells transfected with control siRNA had lamellipodia formation induced 5.3-fold by HDL (Figure 5C), but after knockdown of SR-BI, lamellipodia formation did not occur. Similarly, cells transfected with control siRNA displayed Rac activation by HDL (Figure 5D), and Rac activation was not demonstrable after SR-BI knockdown. As such, SR-BI is required for HDL-induced Rac activation and the resulting changes in the actin cytoskeleton that promote migration.

**HDL and Reendothelialization In Vivo**

To determine whether the mechanisms revealed in vitro are operative in vivo, carotid artery reendothelialization studies were performed in mice. The area of remaining denudation was determined after perivascular electric injury by the injection of Evan’s blue dye, which is incorporated in the region of denudation (supplemental Figure IA and IB).\(^{30,31}\) In control C57BL/6 mice, reendothelialization was complete by 7 days (supplemental Figure IC). Denudation of the endothelium after injury (1 day) and reendothelialization after injury (8 days) was confirmed by immunostaining for vWF (Data...
supplemental Figure ID). To determine the role of HDL in reendothelialization in vivo, reendothelialization was compared 5 days after injury in apoA-I−/− and apoA-I−/+ mice, which have HDL cholesterol levels that are decreased by 83% compared with wild-type. Greater reendothelialization occurred in apoA-I−/+ (Figure 6A) versus apoA-I−/− (Figure 6B) mice, as indicated by the larger area of remaining denudation in apoA-I−/− mice. Cumulative studies revealed that the area of remaining denudation was 52% larger in apoA-I−/− mice versus apoA-I−/+ mice (Figure 6C). Differences in reendothelialization 5 days after injury were confirmed by assessments of endothelial cell density in the region of prior injury by vWF immunostaining (data not shown).

To test whether 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. Five days after injection, apoA-I−/− mice given control adenovirus had HDL levels of 19.6 ± 7.7 mg/dL, whereas mice receiving apoA-I–containing adenovirus had apoA-I levels of 148.4 ± 49.4 mg/dL (P < 0.05 versus control) and HDL levels of 69.7 ± 25.2 mg/dL (P < 0.05 versus control). Evan’s blue dye incorporation 5 days after injury demonstrated that in comparison with apoA-I−/− mice given control adenovirus (Figure 6D), mice injected with apoA-I–containing adenovirus displayed greater reendothelialization (Figure 6D). Quantitation in multiple mice indicated that there was 40% less denudation after the reconstitution of apoA-I expression in apoA-I−/− mice (Figure 6E). These findings indicate that apoA-I and HDL promote endothelial monolayer integrity in vivo.
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<sup>+/−</sup> and SR-BI<sup>−/−</sup> mice 5 days after thermal injury. Greater reendothelialization occurred in SR-BI<sup>+/−</sup> (Figure 7A) versus SR-BI<sup>−/−</sup> mice (Figure 7B) 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<sup>−/−</sup> versus SR-BI<sup>+/−</sup> (Figure 7C). Comparable findings were obtained in older mice studied at 30 to 35 weeks of age (data not shown). Differences in reendothelialization 5 days after injury were confirmed by assessments of endothelial cell density in the region of prior injury by vWF immunostaining (data not shown). Thus, the phenotype of attenuated reendothelialization observed with lowered apoA-I and HDL was recapitulated by loss of SR-BI, thereby providing mechanistic linkage of apoA-I, HDL, and SR-BI in the promotion of endothelial monolayer integrity in vivo.

Because diminished eNOS function causes hypertension<sup>40</sup> and hypertension is associated with endothelial injury,<sup>41</sup> we determined whether the blunted reendothelialization in SR-BI<sup>−/−</sup> mice is related to hypertension. Systolic, diastolic, and mean blood pressure and heart rate measured by radiotelemetry were similar in SR-BI<sup>+/−</sup> and SR-BI<sup>−/−</sup> mice (supplemental Table I). Therefore, the attenuation of reendothelialization in SR-BI<sup>−/−</sup> mice is not due to hypertension.

Because SR-BI<sup>−/−</sup> mice have heterogeneous, enlarged HDL particles with increased cholesterol content compared with those of SR-BI<sup>+/−</sup> mice,<sup>84</sup> it is possible that differences in reendothelialization in SR-BI<sup>+/−</sup> and SR-BI<sup>−/−</sup> are due to disparities in the HDL particle. To address this possibility, BAEC migration responses to human HDL (positive control) or to HDL from SR-BI<sup>+/−</sup> versus SR-BI<sup>−/−</sup> mice were evaluated. Endothelial cell migration with HDL from SR-BI<sup>−/−</sup> mice was at least as robust as that with HDL from SR-BI<sup>+/−</sup> mice (Figure 7D). In studies of lamellipodia formation, responses were identical for HDL from SR-BI<sup>+/−</sup> and SR-BI<sup>−/−</sup> mice (Figure 7E). Thus, the features of HDL required to activate endothelial cell migration are not altered in SR-BI<sup>−/−</sup> mice. In addition, it is important to note that HDL levels in SR-BI<sup>−/−</sup> mice are higher than in wild-type mice,<sup>42</sup> yet reendothelialization is blunted. Therefore, it is not differences in the quantity or nature of the “ligand” for SR-BI that underlie the attenuation in reendothelialization in SR-BI<sup>−/−</sup> mice, and the evidence for a major role for SR-BI in reendothelialization is further strengthened.

Discussion
Circulating levels of HDL and apoA-I are associated with lower risk for vascular disease.<sup>1,2,10,11</sup> In addition, trials with agents such as benzafibrate or gemfibrozil that raise HDL levels indicate that modest elevations in HDL are associated with a significant reduction in overall cardiovascular events.<sup>43,44</sup> Thus, HDL is not simply a marker of decreased vascular disease risk but an important mediator of vascular health. The classical actions of HDL to promote RCT may not fully explain the protective nature of HDL/apoA-I, as RCT is not dictated by circulating levels of HDL or apoA-I.<sup>7–9</sup> In addition, the impact of the antioxidant and antiinflammatory properties of HDL is yet to be clarified.<sup>14,15</sup> In the present study, we show that HDL and SR-BI stimulate endothelial cell migration in vitro with potency equivalent to VEGF. We further demonstrate that the phospholipid, apoA-I, and cholesterol components of HDL are sufficient to initiate this cellular response. Importantly, we also show in the context of all other factors regulating endothelial cell phenotype in vivo that HDL/apoA-I and SR-BI promote endothelial monolayer...
integrity. This represents an entirely novel role for the HDL/SR-BI tandem, complementing the capacity of the lipoprotein and receptor to regulate cholesterol flux and endothelial NO production.3,4,16–19

The processes underlying HDL and SR-BI stimulation of endothelial cell migration were also investigated (Figure 8). Prior studies of HDL activation of eNOS revealed that the lipoprotein activates Src family kinases that activate PI3 kinase, leading to Akt kinase and MAPK stimulation and greater eNOS enzymatic activity.17 In the present study, we demonstrate that the activation of these kinases by HDL–
SR-BI also promotes endothelial cell migration, but that this response is independent of eNOS. 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. We further demonstrate that the kinases reside upstream of Rac in the series of events by which HDL and SR-BI regulate endothelial cell motility (Figure 8, solid arrows). Although antagonism of PI3 kinase or MAPK activity prevented 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 8, dashed arrows). Collectively, these studies have revealed that multiple, sequential signaling events occur in endothelium in response to the lipoprotein. When combined with our prior work on HDL activation of eNOS, they also indicate that after the activation of common upstream signaling events, NO production and cell migration are independently promoted by HDL, thereby enhancing both the integrity and the paracrine functions of the endothelium to optimize vascular health. Whether comparable signaling mediates HDL actions in other cell types is yet to be determined.

In a recent 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 that directly binds cholesterol, and the C-terminal PDZ-interacting domain of SR-BI. In addition, comparable signaling was initiated by HDL and the cholesterol acceptor methyl-beta cyclodextrin, and both were dependent on SR-BI, further suggesting that SR-BI is a cholesterol sensor on the plasma membrane. However, it is yet to be determined how these C-terminal domains of SR-BI, which do not mediate cholesterol movement, initiate signaling. The current work provides in vitro and in vivo evidence that such signaling mediated by HDL and SR-BI is of physiological importance.

The present observations reveal a novel series of mechanisms by which apoA-I/HDL and SR-BI are positive modulators of endothelial cell motility. Our findings provide a new framework for understanding how HDL promotes vascular health. Further research in this realm will enhance both our understanding of HDL and SR-BI signaling and our efforts to harness the potent actions of HDL to develop new strategies to combat vascular disease.

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Dependent processes may also mediate initial lamellipodia and lamellipodia formation, and ultimately to enhanced endothe-
kine and MAPK. These events lead to increased Rac activity sequentially activates Src family kinases, PI3 kinase, and Akt syn-thesis through activation of the phosphatidylinositol 3-kinase/ protein kinase Akt pathway mediates caveolae.

**References**


8. Jolley CD, Woollett LA, Turley SD, Dietschy JM. Centripetal cholesterol flux to the liver is dictated by events in the peripheral organs and not by the plasma high density lipoprotein or apolipoprotein A-I concentration. *J Lipid Res*. 1998;39:2143–2149.


19. Yuhanna IS, Zhu Y, Cox BE, Hahner LD, Osmeño-Lawrence S, Lu P, Marcel YL, Anderson RG, Mendelsohn ME, Hobbs HH, Shaul PW. High-density lipoprotein binding to scavenger receptor-BI activates endo-


27. Kawasaki K, Smith JS, Hsieh CM, Sun J, Chao J, Liao JK. Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates mito-


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Expanded Materials and Methods

Endothelial Cell Migration Assay- BAEC were grown to near-confluency 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 concentrations of HDL studied in all experiments are expressed relative to amount of HDL-associated protein. 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 discoidal lipoprotein Lp2A-1 particles1 (based on amount of apoA-I protein), 1 μmol/L sphingosine-1-phosphate (S-1-P), or 20-50 μg/ml HDL from SR-BI+/+ or SR-BI−/− mice. In other studies, cells were treated with 50 ng/ml pertussis toxin (Ptx), 500 nmol/L PP2, 2 μmol/L LY 294002, 1 μmol/L PD 98059 (Calbiochem), or 2 mmol/L Nω-Nitro-L-arginine methyl ester (L-NAME) (Sigma) and migration responses to 50 μg/ml HDL were tested. Results were confirmed in a minimum of three independent experiments.
Cytoskeletal Changes in Endothelial Cells: BAEC were plated on glass coverslips (Fisher Scientific), grown to 70% confluence and placed in DMEM for 16 h. In selected experiments, cells were pre-treated with 10 μmol/L PP2, 10 μmol/L PD 98059, 20 μmol/L LY 294002, or 2 mmol/L L-NAME for 30 min. 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. 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. In selected experiments, cells were pre-treated with 10 μmol/L PP2, 10 μmol/L PD 98059 or 20 μmol/L LY 294002 for 30 min and treated with 50 μg/ml HDL for 5 min. After treatment, Rac activity was measured by binding of Rac to the fragment of p21-activated kinase (PAK) containing the Rac-binding domain conjugated to glutathione-S-transferase (GST-PAK) as previously described with minor modifications. GST-PAK was provided by Dr. F. Grinnell, UT Southwestern. Active and total Rac were detected by immunoblot analysis with anti-Rac antibody (Upstate Biotechnology). Results were confirmed in four independent experiments.
Preparation of HDL- Human HDL prepared by density gradient ultracentrifugation\(^3\) was provided by Drs. J. Goldstein and M. Brown, UT Southwestern. The HDL was isolated from healthy volunteers after a 12h fast and the HDL\(_{3}\) subfraction (density range 1.12-1.2181 g/ml) was obtained and used in all experiments. The HDL was stored at 4\(^\circ\)C prior to use. The HDL fraction from SR-BI\(^{+/+}\) or SR-BI\(^{-/-}\) mice was isolated according to procedures described previously\(^4\).

Preparation of Reconstituted HDL Particles- Homogeneous lipoprotein Lp2A-I particles were prepared from 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), cholesterol, and lipid-free recombinant apoA-I at molar ratios of 40:0:1, 80:0:1, or 80:5:1 as described previously\(^1\). Briefly, the method entails the incubation at 37\(^\circ\)C under nitrogen of POPC, free (unesterified) cholesterol (FC), cholate and apoA-I in proper stoichiometric amounts. Following removal of cholate with Biobeads, the preparation was dialyzed. The homogeneity of the particles was confirmed by non-denaturing gradient gel electrophoresis and compositions of the final products were determined enzymatically (for POPC and FC) and by a modification of the Lowry method (for apoA-I). The Lp2A-1 particles were all approximately 7 to 8 nm in diameter\(^1,5,6\). For Lp2A-I with desired molar ratios of POPC:cholesterol:apoA-I of 80:0:1 the final actual composition was 71-87:0:1, for 40:0:1 the final actual composition was 35-47:0:1, and for 80:5:1 the final actual composition was 73-88:4-5:1. In preliminary experiments lipid-free apoA-I alone did not activate endothelial cell migration.
siRNA Preparation and Transfection: dsRNAs with sequences 5’-UGCGUUUCGGGAGAAUAU-3’ and 5’-UCCGAGCAAGAAUGTT-3’ were designed to target the open reading frames of bovine Rac1 and bovine SR-BI. A dsRNA with sequence 5’-AGUUAGACCAGCGAGGATT-3’ served as control (control siRNA). BAEC were transfected with 40 nmol/L RNA as described previously. 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.

Immunoblot Analyses: Rac abundance in Rac activity assays or following siRNA was determined by immunoblot analysis with anti-Rac antibody (Upstate). SR-BI protein abundance after siRNA was assessed using anti-SR-BI antibody (provided by Dr. H. Hobbs, UT Southwestern). For kinase activation experiments, cells were treated with 50 μg/ml HDL for 5 or 10 min, harvested, and lysates were subjected to SDS-PAGE and immunoblot analysis using antibodies to phospho-Src (Cell Signaling), c-Src (Santa Cruz), phospho-Akt, Akt, phospho-MAPK (Cell Signaling) and MAPK (Upstate).

Carotid Artery Reendothelialization: Carotid artery reendothelialization was studied following perivascular electric injury 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). Mice were anesthetized by intraperitoneal administration of avertin (combination of 40 ml 2.5% tribromoethanol and 310 μl tertiary amylalcohol in 39.5 ml water) at 0.02-0.04ml/g body weight. Additional experiments were performed in 30-35 week-old SR-BI^{+/+} versus SR-BI^{-/-} mice. Liver directed gene transfer of apoA-I was performed in apoA-I^{-/-} mice by intravenous administration of control or human apoA-I-containing adenovirus (1X10^{11} particles per mouse), and apoA-I and HDL levels were measured 5 d after injection. 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.

**Immunohistochemistry:** Carotid arteries of 10-15 week old male C57BL/6 mice were subjected to perivascular electric injury. Arteries of uninjured, 1-d and 8-d injured mice were harvested and sectioned into 7 μm-thick sections superior, within and inferior to the region of injury. Sections were stained with 16.6 μg/ml anti-von Willebrand Factor (VWF) antibody (Abcam), 2 μg/ml biotinylated-secondary antibody, 2.5 μg/ml HRP-streptavidin (Kirkegaard and Perry Laboratories), and diaminobenzidine (Sigma) and viewed by light microscopy at
400X magnification. The same approach was used in studies of reendothelialization in wild type, apoA-I\(^{-/-}\), and SR-BI\(^{-/-}\) mice.
Supplemental Figure 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. (D) Confirmation of endothelial denudation and reendothelialization postinjury. Arteries subjected to sham procedure or denudation were sectioned (7 μm) through the area of injury on d 1 or d 8 postinjury, and immunohistochemistry for von Willebrand Factor (vWF) was performed. Arrows indicate vWF staining of endothelial cells. Sections were viewed by light microscopy at 400X magnification.
### Supplemental Table

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

Blood pressure and heart rate are similar in SR-BI^{+/+} and SR-BI^{-/-} mice. Values are mean±SEM.
References


11. Tsukamoto K, Hiester KG, Smith P, Usher DC, Glick JM, Rader DJ. Comparison of human apoA-I expression in mouse models of