The Scavenger Receptor Class B Type I Adaptor Protein PDZK1 Maintains Endothelial Monolayer Integrity

Weifei Zhu, Sonika Saddar, Divya Seetharam, Ken L. Chambliss, Christopher Longoria, David L. Silver, Ivan S. Yuhanna, Philip W. Shaul,* Chieko Mineo*  

Abstract—Circulating levels of high-density lipoprotein (HDL) cholesterol are inversely related to the risk of cardiovascular disease, and HDL and the HDL receptor scavenger receptor class B type I (SR-BI) initiate signaling in endothelium through src that promotes endothelial NO synthase activity and cell migration. Such signaling requires the C-terminal PDZ-interacting domain of SR-BI. Here we show that the PDZ domain–containing protein PDZK1 is expressed in endothelium and required for HDL activation of endothelial NO synthase and cell migration; in contrast, endothelial cell responses to other stimuli, including vascular endothelial growth factor, are PDZK1-independent. Coimmunoprecipitation experiments reveal that Src interacts with SR-BI, and this process is PDZK1-independent. PDZK1 also does not regulate SR-BI abundance or plasma membrane localization in endothelium or HDL binding or cholesterol efflux. Alternatively, PDZK1 is required for HDL/SR-BI to induce Src phosphorylation. Paralleling the in vitro findings, carotid artery reendothelialization following perivascular electric injury is absent in PDZK1−/− mice, and this phenotype persists in PDZK1−/− mice with genetic reconstitution of PDZK1 expression in liver, where PDZK1 modifies SR-BI abundance. Thus, PDZK1 is uniquely required for HDL/SR-BI signaling in endothelium, and through these mechanisms, it is critically involved in the maintenance of endothelial monolayer integrity. (Circ Res. 2008;102:480–487.)  

Key Words: PDZK1 ■ high-density lipoprotein ■ SR-BI ■ endothelium

The risk of atherosclerosis is inversely related to circulating high-density lipoprotein (HDL) cholesterol levels,1,2 and there is also evidence that a lower HDL level is associated with a greater likelihood of restenosis after a vascular intervention.3,4 HDL classically functions in reverse cholesterol transport, removing cholesterol from peripheral tissues and delivering it to the liver and to steroidogenic organs by binding of the major HDL apolipoprotein, apolipoprotein (apo)A-I, to the high-affinity HDL receptor scavenger receptor B type I (SR-BI).5,6 In mouse models of atherosclerosis, apoA-I and SR-BI both provide atheroprotection,7,8 and in the context of experimental hypercholesterolemia, the provision of apoA-I or HDL attenuates neointima formation after artery injury.9,10 The protective nature of HDL has been previously attributed to its role in reverse cholesterol transport. However, evidence is accumulating that HDL has a number of additional actions that also afford cardiovascular protection, and many of these entail direct modulation of endothelial cell phenotype.11

The direct actions of HDL on the endothelium are multiple. In particular, HDL promotes the production of the atheroprotective signaling molecule NO by upregulating endothelial NO synthase (eNOS) expression,12 by maintaining the lipid environment in caveolae, where eNOS is colocalized with partner signaling molecules,13 and by stimulating eNOS enzymatic activity.14,15 As importantly, HDL protects endothelial cells from apoptosis and promotes their growth and migration,11,16 thereby maintaining the integrity of the endothelial monolayer. The direct actions of HDL on endothelium are mediated by SR-BI, which is enriched in endothelial cell caveolae and required for signaling by the lipoprotein.14,16 The latter processes entail initial Src activation, which results in parallel activation of Akt kinase and mitogen-activated kinases.15 These events then lead to eNOS stimulation and to the activation of Rac GTPase, which initiates endothelial cell migration in an NO-independent manner.15,16 The most proximal events involve cholesterol flux, the C-terminal transmembrane domain of SR-BI that directly binds cholesterol, and the C-terminal PDZ-interacting domain of SR-BI.17 A major role for apoA-I/HDL and SR-BI in the maintenance of endothelial monolayer integrity has been previously demonstrated in vivo in mice.16 However, the molecular basis for the coupling of SR-BI to downstream events governing endothelial cell behavior is unknown.

Recognizing the absolute requirement for the C-terminal PDZ-interacting domain of SR-BI in signal initiation,17 the...
present study investigated the potential role of the multi-PDZ domain–containing adaptor protein PDZK1 in the vascular actions of the HDL/SR-BI tandem. Studies in the liver first indicated that PDZK1 binds directly to the C terminus of SR-BI, and further work has shown that it mediates hepatic SR-BI levels. We raised the hypothesis that PDZK1 is expressed in endothelial cells and necessary for the modulation of endothelial cell phenotype by HDL and SR-BI. We determined the role of PDZK1 in HDL-induced eNOS activation, and in HDL-mediated endothelial cell migration, which is NO-independent. Additional experiments were designed to reveal how PDZK1 governs SR-BI function in endothelium. Furthermore, carotid artery reendothelialization was assessed after perivascular electric injury in mice in which PDZK1 status was genetically manipulated to reveal whether PDZK1 participates in the maintenance of intimal layer integrity.

Materials and Methods

Animal Model
Experiments were performed in littermate wild-type PDZK1+/- mice and PDZK1-/- mice in which exon 1 and a portion of intron 1 of the PDZK1 allele were replaced by the Neo cassette as described previously.

Cell Culture
Bovine aortic endothelial cells (BAECs) were harvested using procedures reported previously with minor modifications, cultured in EGM-2 medium (Cambrex Corp) with 5% FBS (Sigma-Aldrich), and studied at passages 5 to 9. Additional experiments were performed in primary mouse endothelial cells grown from explants of aortas from PDZK1+/- mice.

Immunoblot Analysis
PDZK1, SR-BI, eNOS, caveolin-1, c-Src, and actin protein abundance was evaluated using established procedures. Additional details are provided in the online data supplement.

Modification of PDZK1 in Cultured Endothelial Cells
To knock down PDZK1 expression, a small interfering (si)RNA-based strategy was used. Double-stranded RNA sequences directed against PDZK1 were transfected into cells, and expression of PDZK1 and SR-BI and functional readouts were determined 24 to 48 hours later. To enhance PDZK1 expression, an adenoviral construct encoding murine PDZK1 was used. Additional details are provided in the online data supplement.

eNOS Activation Assays
eNOS activation was assessed in whole cells by measuring [14C]-L-arginine conversion to [14C]-L-citrulline. Cell treatments included HDL (10 μg/mL), acetylcholine (10 μmol/L), or vascular endothelial growth factor (VEGF) (1.2 pmol/L or 50 ng/mL). Additional details are provided in the online data supplement. eNOS activation was also evaluated over 60 minutes in isolated endothelial cell plasma membranes in the presence of added calcium, calmodulin, or eNOS cofactors, with HDL (10 μg/mL) as the stimulus. This model system allows interrogation of the participating signaling molecules by antibody blockade. To test the role of PDZK1 in HDL signaling, experiments were performed in the absence or presence of 500 μg/mL of monoclonal antibody to PDZK1, which was kindly provided by Hiroyuki Arai (Department of Health Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, Japan). Control treatment was with unrelated IgG, and results were confirmed in 3 independent experiments.

Endothelial Cell Migration Assay
BAECs were grown to near confluence, and a defined region of cells was removed with a razorblade. Cells were treated for 24 hours, fixed, and the number of cells that had migrated past the wound edge was quantified. Additional details are provided in the online data supplement.

HDL Binding and Cholesterol Efflux
Details regarding measurements of HDL binding and cholesterol efflux from BAECs are provided in the online data supplement.

Coimmunoprecipitation
Details regarding SR-BI and coimmunoprecipitation are provided in the online data supplement.

Carotid Artery Reendothelialization
Carotid artery reendothelialization was studied following perivascular electric injury in mice by assessing Evans blue dye uptake. Study groups included wild-type PDZK1+/- mice, PDZK1-/- mice, and PDZK1-/- mice with adenoviral reconstitution of hepatic PDZK1 expression.

Statistical Analysis
All data are presented as means±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

PDZK1 Expression and Subcellular Localization
Vascular PDZK1 expression was first evaluated in whole mouse aorta. Whereas the protein was detected in aortas from wild-type PDZK1+/- mice, it was not detected in aortas from PDZK1-/- mice that served as a negative control (Figure 1A). PDZK1 was also detected in primary endothelial cells cultured from explants of aortas from wild-type mice (Figure 1B).

The subcellular localization of PDZK1 was evaluated in the postnuclear supernatant, cytoplasmic, and plasma membrane fractions of BAECs (Figure 1C). Whereas SR-BI protein was concentrated in plasma membranes, PDZK1 was detected in the cytoplasm and in the plasma membrane fraction.

Role of PDZK1 in eNOS Activation
To determine whether PDZK1 is required for HDL-mediated signaling in endothelium, eNOS activation was evaluated in cells in which PDZK1 expression was knocked down by siRNA (Figure 2A). Whereas HDL caused eNOS stimulation in cells transfected with control siRNA, signaling to eNOS by HDL was not evident in cells transfected with PDZK1 siRNA (Figure 2B). In contrast, the loss of PDZK1 expression did not alter eNOS activation by acetylcholine or VEGF, indicating that the siRNA effect is specific to PDZK1 and that PDZK1 is uniquely required for signaling by HDL.

To provide an independent means to determine whether PDZK1 is necessary for signal initiation by HDL, antibody blockade was performed during measurements of eNOS activation by HDL in isolated endothelial plasma membranes. In prior work, antibody to the C-terminal cytoplasmic tail of SR-BI fully attenuated eNOS activation by HDL in isolated plasma membranes. Paralleling the previous findings,
monoclonal antibody to PDZK1 prevented eNOS activation by HDL, whereas unrelated IgG had no effect (Figure 2C). Therefore, PDZK1 is both in the plasma membrane fraction and the cytoplasm of endothelial cells, and the subpopulation of the protein associated with the plasma membrane is required for HDL signaling to eNOS.

Role of PDZK1 in Cell Migration
Along with its capacity to activate eNOS, we have previously demonstrated that HDL promotes endothelial cell migration via SR-BI in an NO-independent manner. To determine whether PDZK1 is required for this process, endothelial cells were transfected with control siRNA or PDZK1 siRNA to knock down expression of the protein, wounded, and treated with HDL or VEGF. In control cells the lipoprotein caused a marked increase in migration that was comparable to that stimulated by VEGF (Figure 3A). In contrast, cells transfected with PDZK1 siRNA had diminished migration in response to HDL, whereas VEGF-induced migration was unaltered. Summary data from 4 experiments indicated that PDZK1 siRNA blunted HDL-induced migration by 60% to 66% (Figure 3B). These findings indicate that HDL-induced endothelial cell migration is PDZK1-dependent, whereas the response to VEGF is PDZK1-independent.

Impact of PDZK1 on SR-BI
To understand the basis for the specific requirement for PDZK1 in HDL modulation of endothelial cell phenotype, the impact of PDZK1 on vascular and endothelial SR-BI expression was determined. As has been reported previously, a lack of PDZK1 in the liver resulted in a marked decline in SR-BI protein abundance (Figure 4A). In contrast, SR-BI expression was similar in aortas from PDZK1+/+ and PDZK1−/− mice. To specifically investigate the role of PDZK1 in the regulation of SR-BI expression in endothelial cells, PDZK1 was knocked down by siRNA and immunoblot analysis for SR-BI was performed. Receptor expression was unchanged with the loss of PDZK1 in endothelial cells (Figure 4B). To provide a complementary approach, PDZK1 was overexpressed in endothelial cells using an adenoviral construct, and SR-BI abundance remained unchanged (Figure 4C).

Because PDZK1 serves as an adaptor protein for selected signaling molecules in nonendothelial cells impacting not only their abundance but their membrane association and function, the role of PDZK1 in directing SR-BI subcellular localization in endothelial cells was investigated. In control cells, SR-BI was enriched in the endothelial cell plasma membrane as previously described (Figure 4D). Knockdown of PDZK1 by siRNA had no effect on SR-BI localization.
association with the plasma membrane. In addition, the plasma membrane enrichment with eNOS was similar in control cells and cells transfected with PDZK1 siRNA.

The participation of PDZK1 in HDL binding to SR-BI and cholesterol efflux in endothelial cells was also evaluated. 125I-HDL binding to BAECs was unaffected by knockdown of PDZK1 by siRNA (Figure 4E). Specific binding of HDL (125I-HDL binding not displaced by unlabeled HDL) was similarly attenuated by antibody to the SR-BI extracellular domain in control and PDZK1 siRNA-treated endothelial cells, being decreased by 72±4% and 64±4%, respectively. In addition, cholesterol efflux to HDL was unchanged by the loss of PDZK1 (Figure 4F). These cumulative observations indicate that PDZK1 does not modify SR-BI abundance, its subcellular targeting, or its classical role in the regulation of cholesterol flux in endothelial cells.

The most proximal signaling event known to be initiated by HDL/SR-BI in endothelium is the activation of Src family kinases. To determine whether PDZK1 plays a role in HDL/SR-BI-induced Src activation, Src phosphorylation in response to HDL was assessed in cells with normal versus diminished PDZK1 expression using antibodies to c-Src. In cells transfected with control siRNA, HDL caused Src phosphorylation at 10 and 15 minutes of treatment (Figure 5A). In contrast, in cells transfected with PDZK1 siRNA, HDL did not cause Src activation. Cumulative results indicated that there was a 3-fold increase in Src phosphorylation with HDL treatment that was entirely PDZK1-dependent (Figure 5B). The requirement for SR-BI interaction with PDZK1 in HDL/SR-BI activation of Src was then determined by the transfection of a hemagglutinin-tagged, truncated form of PDZK1 comprised of amino acids 1 to 240 (TR-PDZK1), which consists of the 2 N-terminal PDZ domains including the interaction domain with SR-BI and lacks the 2 C-terminal PDZ domains (Figure 5C). Overexpressed TR-PDZK1 would therefore compete with endogenous wild-type PDZK1 for binding to endogenous SR-BI. Whereas sham-transfected cells displayed Src phosphorylation in response to HDL, cells

Figure 3. PDZK1 is required for HDL-induced endothelial cell migration. A, BAECs were transfected with control siRNA or PDZK1 siRNA, and 24 hours later, the cells were wounded and treated with media alone (control), media plus HDL, or media plus VEGF, and migration was evaluated over 18 hours. B, Summary data for control cells (open bar) and PDZK1 siRNA cells (closed bar) from 3 experiments. Values are means±SEM. *P<0.05 vs control siRNA.

Figure 4. PDZK1 does not regulate SR-BI expression or subcellular localization or HDL binding to SR-BI or cholesterol efflux to HDL in endothelium. A, PDZK1 and SR-BI abundance was evaluated in the livers and thoracic aortas of PDZK1+/− and PDZK1−/− mice by immunoblot analysis. B, BAECs were transfected with control siRNA or PDZK1 siRNA, and PDZK1 and SR-BI abundance was evaluated 24 hours later by immunoblot analysis. C, BAECs were transfected with control adenovirus or adeno-PDZK1, and PDZK1 and SR-BI abundance was evaluated 48 hours later by immunoblot analysis. In B and C, results with duplicate samples are shown. PDZK1 was detectable in control samples with longer immunoblot exposure (data not shown). D, BAECs were transfected with control siRNA or PDZK1 siRNA, 24 hours later postnuclear supernatant (PNS), cytoplasm, and plasma membrane (PM) fractions were prepared, and SR-BI and eNOS abundance in the subfractions was evaluated by immunoblot analysis. Knockdown of PDZK1 was confirmed in PNS samples. Results shown in A through D are representative of 3 independent experiments. E, BAECs were transfected with control siRNA or PDZK1 siRNA, and 125I-HDL binding in the absence or presence of excess unlabeled HDL was evaluated 48 hours later. Values are means±SEM (n=3). *P<0.05 vs no unlabeled HDL. F, BAECs were transfected with control siRNA or PDZK1 siRNA and loaded for 24 hours with 3H-cholesterol beginning 24 hours later, and cholesterol efflux to HDL was then measured over 360 minutes. Values are means±SEM (n=3).
expressing TR-PDZK1 did not. Summary findings revealed that the capacity of endogenous, wild-type PDZK1 to mediate a 3-fold increase in Src phosphorylation in response to HDL was fully inhibited by TR-PDZK1 (Figure 5D).

The basis by which PDZK1 enables HDL/SR-BI to activate Src was further investigated in coimmunoprecipitation experiments. BAECs transfected with SR-BI cDNA such that the receptor could be readily immunoprecipitated were cotransfected with either PDZK1 cDNA or the siRNA targeting PDZK1 to yield abundant versus absent PDZK1 (Figure 5E, left). Twenty-four hours later, immunoprecipitation was performed on postnuclear supernatants with anti–SR-BI antibody directed to the extracellular domain (+ lane) or unrelated IgG (- lane), and immunoblotting for SR-BI and Src was performed on the immunoprecipitates (right). Results shown were replicated in 2 independent experiments.

Role of PDZK1 In Vivo

To determine whether PDZK1 modulates endothelial cell phenotype in vivo, carotid artery reendothelialization studies were performed in PDZK1+/+ and PDZK1−/− mice. The area of remaining denudation was determined after perivascular electric injury by the injection of Evans blue dye, which is incorporated in the region of denudation. On the day of injury, the area of initial denudation was similar in PDZK1+/+ and PDZK1−/− mice (Figure 6A, top images). At 5 days postinjury, markedly less reendothelialization had occurred in PDZK1−/− versus PDZK1+/+ mice, as indicated by the larger area of remaining denudation (Figure 6A, bottom images). Cumulative studies revealed 79% reendothelialization in PDZK1+/+ mice and an absence of reendothelialization in PDZK1−/− mice (Figure 6B).

To determine whether the loss of reendothelialization in PDZK1−/− mice is attributable to absence of the protein in the liver, hepatic expression of PDZK1 was rescued in PDZK1−/− mice by liver-directed gene transfer of PDZK1 before artery injury. In mice receiving PDZK1-containing adenovirus, both PDZK1 and SR-BI expression in the liver were rescued to levels observed in wild-type mice (Figure 7A), and this resulted in a normalization of circulating total cholesterol (Figure 7B). In contrast, vascular PDZK1 remained undetectable (Figure 7C). With hepatic rescue of PDZK1 expression and function, the impaired reendothelialization phenotype in PDZK1−/− mice was unaffected (Figure 7D and 7E), providing additional evidence...
the enzyme by both a G protein–coupled receptor agonist, acetylcholine, and a growth factor, VEGF, are unaltered. In prior work, we demonstrated that HDL activation of eNOS in isolated endothelial cell plasma membranes is fully blocked by the addition of an antibody to the C terminus of SR-BI,14 thereby providing the first evidence of involvement of this domain of the receptor in HDL signaling. In the same model system, we now demonstrate that monoclonal antibody to PDZK1 also attenuates eNOS activation by HDL in the isolated plasma membranes, whereas an unrelated antibody does not. The finding that PDZK1 is required for HDL activation of eNOS is in agreement with a recent report showing that PDZK1 knockdown in cultured human umbilical vein endothelial cells (HUVECs) attenuates HDL-mediated effects via SR-BI. The present observations extend beyond the prior report by indicating that plasma membrane-associated PDZK1 is required for HDL activation of eNOS and importantly that there is a specific role for PDZK1 in HDL/SR-BI signaling to eNOS and not signaling by other classic ligand–receptor pairs that activate the enzyme.

To determine the requirement for PDZK1 in the regulation of a key endothelial cell behavior that is governed by HDL/SR-BI but not eNOS,16 HDL-induced migration was studied in BAECs transfected with control siRNA or PDZK1 siRNA. The migratory response to HDL, which was comparable in degree to that obtained with VEGF, was markedly attenuated following the knockdown of PDZK1. The resulting change in phenotype parallels that previously observed with depletion of endothelial cell SR-BI by siRNA.16 In contrast, endothelial cell migration with VEGF was not affected in PDZK1-depleted cells. These observations provide additional evidence that PDZK1 is specifically required for HDL/SR-BI modulation of endothelial cell phenotype.

The basis for the requirement for PDZK1 in HDL/SR-BI actions in endothelial cells was then investigated. In hepatocytes, PDZK1 modulates the steady-state levels of SR-BI19,30 and potentially similar mechanisms in vascular cells were evaluated. Whereas SR-BI abundance was markedly lower in livers of PDZK1−/− mice versus PDZK1+/+ mice, levels in whole aorta were unchanged, suggesting that the mechanisms regulating SR-BI expression are different in these tissues. In addition, neither the knockdown nor overexpression of PDZK1 in cultured endothelial cells altered SR-BI expression. These findings indicate that PDZK1 does not regulate SR-BI abundance in endothelial cells. Potential involvement of PDZK1 in SR-BI subcellular localization was then investigated, and it was found that SR-BI targeting to the plasma membrane of endothelial cells was not modified by the loss of PDZK1. Importantly, eNOS targeting to the plasma membrane was also not regulated by PDZK1. In addition, HDL binding to endothelial cell SR-BI and cholesterol efflux to HDL were not PDZK1-dependent.

The participation of PDZK1 in the coupling of SR-BI to the most proximal signaling event activated by HDL/SR-BI in endothelium, namely Src activation,15 was then investigated. Both PDZK1 knockdown by siRNA and the overexpression of a truncated form of PDZK1 (TR-PDZK1) including the region that interacts with SR-BI caused complete attenuation of Src phosphorylation in response to HDL. The dominant-negative effect of TR-PDZK1, which is capable of

**Figure 6.** Carotid artery reendothelialization is absent in PDZK1−/− mice. A, The intimal surface of Evans blue-stained arteries from PDZK1+/+ and PDZK1−/− mice are shown 1 day (D1) and 5 days (D5) after injury. B, Area of denudation was quantified and expressed in arbitrary units. Values are means±SEM (n=8 mice per group). *P<0.05 vs day 1, †P<0.05 vs PDZK1+/+. Supporting a key role of endothelial PDZK1 in the maintenance of an intact endothelial monolayer.

**Discussion**

Circulating levels of HDL cholesterol are associated with lower risk for cardiovascular disease. Along with its role in mediating reverse cholesterol transport, HDL has multiple endothelial actions that also afford cardiovascular protection. These include the activation of eNOS, protection from apoptosis, and the promotion of endothelial cell growth and migration. HDL signaling in endothelium is mediated by the high-affinity HDL receptor SR-BI.11 Here we show that HDL modulation of diverse endothelial cell phenotypes via SR-BI requires the adaptor molecule PDZK1. Thus, PDZK1 plays a central role in the promotion of vascular health by HDL.

In studies of mouse aorta, we first demonstrate that PDZK1 is expressed in vascular cells in vivo. Lack of detection of the protein in aortas from PDZK1−/− mice serves as an important negative control for detection of a protein that is primarily comprised of PDZ domains. PDZK1 protein is also detected in primary mouse endothelial cells, supporting the use of the mouse to interrogate the vascular actions of PDZK1 in vivo. In contrast to SR-BI, which is concentrated in endothelial caveola membranes, PDZK1 was found to be both membrane-associated and in the cytoplasm of endothelial cells. Thus, PDZK1 is expressed and localized in endothelial cells, where it can potentially mediate the actions of HDL/SR-BI.

The role of PDZK1 in endothelial function was then initially assessed in studies of eNOS activation. We show that the loss of PDZK1 from endothelial cells by siRNA causes complete prevention of HDL activation of eNOS, whereas stimulation of
interaction with SR-BI but lacks the 2 C-terminal PDZ domains, suggests that endogenous PDZK1 association with SR-BI and regions within the C terminus of the adaptor protein are required for the signaling to Src invoked by SR-BI. Coimmunoprecipitation experiments further showed for the first time that Src interacts with SR-BI, and whereas Src activation by HDL/SR-BI is entirely PDZK1-dependent, the association of Src and SR-BI is not. These findings suggest that PDZK1 modifies the localization or function of kinase(s) required for Src phosphorylation by HDL/SR-BI. Detailed studies targeting the 2 C-terminal PDZ domains of PDZK1 and potentially associated kinase(s) are now indicated to determine the molecular basis of the requirement for PDZK1 in src regulation by HDL/SR-BI.

To determine whether PDZK1 modulates endothelial cell behavior in vivo, carotid artery reendothelialization studies were performed in PDZK1−/− versus PDZK1+/+ mice. In contrast to findings in wild-type mice, reendothelialization was absent in PDZK1−/− mice, paralleling the phenotype we previously demonstrated in apoA-I−/− mice and in SR-BI−/− mice. The impairment in reendothelialization was also apparent in PDZK1−/− mice with genetic rescue of PDZK1 in the liver and resulting rescue of hepatic SR-BI and normalization of circulating cholesterol levels, providing additional evidence supporting an important role for endothelial PDZK1 and SR-BI in vascular health. Furthermore, because the in vitro studies of endothelial cell migration showed specificity of PDZK1 involvement in HDL-mediated endothelial cell migration, these collective findings reveal that in the context of all other factors regulating endothelial cell phenotype, the molecular pathway comprised of HDL/apoA-I, SR-BI, and now PDZK1 is likely a major promoter of endothelial monolayer integrity in vivo. With these multiple observations now in hand, in vivo studies of the impact of conditional expression of PDZK1 in endothelium are worthy of pursuit.

The present observations reveal a key role for the adaptor protein PDZK1 in the modulation of endothelial cell phenotype by apoA-I/HDL and SR-BI. Our findings provide a new mechanistic context for understanding specifically how HDL initiates signal transduction and generally how HDL has beneficial impact on vascular health. Further research on the vascular biology of PDZK1 will enhance our capacity to apply the potent actions of HDL to prevent and combat cardiovascular disease.

Acknowledgments

We are indebted to Dr Daniel Rader for critical assessment of the manuscript.

Sources of Funding

This work was supported by NIH grants HL58888 (to P.W.S.) and HL082697 (to D.L.S.). Additional support was provided by the Crystal Charity Ball Center for Pediatric Critical Care Research and the Lowe Foundation (to P.W.S.).

Disclosures

None.

References

The Scavenger Receptor Class B Type I Adaptor Protein PDZK1 Maintains Endothelial Monolayer Integrity

Weifei Zhu, Sonika Saddar, Divya Seetharam, Ken L. Chambliss, Christopher Longoria, David L. Silver, Ivan S. Yuhanna, Philip W. Shaul and Chieko Mineo

*Circ Res.* 2008;102:480-487; originally published online January 3, 2008; doi: 10.1161/CIRCRESAHA.107.159079

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/102/4/480

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/01/03/CIRCRESAHA.107.159079.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the *Permissions and Rights Question and Answer* document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/
Online Supplement

Expanded Materials and Methods

**Immunoblot Analyses:** The methods used for immunoblot analysis generally followed those previously reported using ECL reagents for chemiluminescence (Amersham Biosciences). Primary antibodies were from the following sources: anti-PDZK1 monoclonal antibody was from Abnova Corp., anti-PDZK1 polyclonal antibody was from Affinity BioReagents, anti-SR-BI polyclonal antibody was from Novus Biologicals, Inc., anti-eNOS and anti-caveolin-1 monoclonal antibodies were from BD Transduction Laboratories, anti-src monoclonal antibody and anti-actin polyclonal antibody were from Santa Cruz Biotechnology, Inc. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were also from Santa Cruz. In selected studies endothelial cells were subfractionated as previously described. For Src activation experiments, cells were treated with 50 μg/ml HDL for 0 to 20 min, and immunoblot analyses were performed using antibody to phospho-tyrosine-416-c-Src (Cell Signaling Technology). Immunoblotting for total c-Src was also performed (B-12, Santa Cruz), and the relative abundance of phosphorylated Src was calculated. In selected experiments, the cells were transfected with cDNA encoding an HA-tagged truncated form of PDZK1 (aa 1-240) for 48 h before they were treated with HDL.

**siRNA Preparation and Transfection:** dsRNAs with sequences 5’-CAGCCAGUUUGAACUGCUUU-3’ and 5’-AGCAGUUUCAACUGGCUGUU-3’ were designed to target the open reading frames of bovine PDZK1. A dsRNA
with sequence 5'-AGUUAGACCAGACCGAGGATT-3' served as control. BAEC were transfected with 60 nmol/L RNA as described previously. 24h after transfection, NOS activation was evaluated in intact cells, the cells were placed in DMEM with 1% LPDS for 16 h for migration studies, or they were treated with HDL (50µg/ml) for 0-20 min for src phosphorylation experiments. To enhance endothelial cell PDZK1 expression BAEC were infected with sham adenovirus or with an adenoviral construct encoding murine PDZK1 (Viraquest). Cells were grown to 80% confluence in 60-mm dishes and placed in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich) containing 2% serum, adenovirus (50 PFU/cell) was added for 2h, the media was changed, and experiments were performed 48h later.

**Cell Fractionations:** To study the subcellular distribution of PDZK1, subfractionation was performed on transfected BAEC as previously described. The purity of the PM fraction obtained has been previously confirmed by measurements of alkaline phosphatase (PM), galactosyl transferase (Golgi) and reduced nicotinamide adenine dinucleotide phosphate cytochrome C reductase (endoplasmic reticulum) activity. Successful isolation of the plasma membrane was confirmed by immunoblot analyses for caveolin-1 and SR-BI.

**eNOS Activation Assays:** Enzyme stimulation was assessed in intact cells in PBS containing 120 mmole/L NaCl, 4.2 mmole/L KCl, 2.5 mmole/L CaCl$_2$, 1.3 mmole/L MgSO$_4$, 7.5 mmole/L glucose, 10 mmole/L Hepes, 1.2 mmole/L Na$_2$HPO$_4$, and 0.37 mmole/L KH$_2$PO$_4$ at pH 7.4. Following a 15 min
preincubation, radiolabeled arginine was added and the 15 min incubation for eNOS activation was performed in the absence (basal) or presence of agonist. HDL (50 μg/ml), Ach (10^{-5} M) and VEGF (50 ng/ml) yielded similar eNOS activation to levels that were 201±25%, 170±25% and 204±29% of basal activity, respectively.

**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-Aldrich) for 16 h, and a defined region of cells was removed with a single-edged razor blade. Cells were treated with HDL (20 or 50 μg/ml) in DMEM + 1% LPDS and 24 h later fixed with 3% paraformaldehyde (Sigma-Aldrich), 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 parallel studies, cells were treated with 50 ng/ml vascular endothelial growth factor (VEGF) (Calbiochem). Results were confirmed in a minimum of three independent experiments.

**HDL Binding**: BAEC were transfected with control double-stranded RNA (dsRNA) or dsRNA directed at bovine PDZK1. 48h after transfection, cells were
washed with DMEM containing 0.5% BSA, and an additional set of cells was pre-
incubated with an antibody to SR-BI that recognizes the extracellular domain and
blocks HDL binding (NOVUS, 2 μg/ml) for 30 min. 125I-HDL (10 μg/ml) prepared
using Bolton-Hunter reagent (Perkin Elmer) was added in the presence or
absence of excess unlabeled HDL (200 μg/ml), and the cells were incubated for
90 min at 4°C. The cells were washed with PBS, lysed in 0.1N NaOH, and 125I-
HDL associated with the cells was measured by γ-counter. The protein
concentration was determined by the DC protein assay (BioRad).

Cholesterol Efflux 10: BAEC were transfected with control double-stranded RNA
dsRNA) or dsRNA directed at bovine PDZK1. 24h after transfection, the cells
were labeled with 3H-cholesterol (Perkin Elmer, 2 μCi/ml) in the presence of
Sandoz 58-035 (ACAT inhibitor, 1 μg/ml) for 24h. The cells were washed with
DMEM containing 1% BSA, acceptor medium containing 0, 25 or 250 μg/ml HDL
was added, the cells were incubated at 37°C, and 100 ul of culture medium was
collected at 60, 120, 240 and 360min. The cells were washed with PBS and total
cellular lipids were extracted and counted by liquid scintillation. Efflux of
cholesterol was calculated as a fraction of the total radioactive cellular
cholesterol.

Coimmunoprecipitation: BAEC were cotransfected with cDNAs for SR-BI and
PDZK1-HA (C-terminal HA tag) or cDNA for SR-BI and PDZK1 siRNA. Cells
were harvested 24h after transfection and post-nuclear supernatant (PNS) was
isolated. 400 µg of PNS was dissolved in buffer A (0.25 M sucrose, 1 mM EDTA and 20 mM Tricine) and RIPA buffer (100 mM Tris pH 7.4, 300 mM NaCl, 2 % NP-40, 0.5 % Na-deoxycholate and 2 mM EDTA) (mixed in equal volume) and the samples were precleared with protein A/G Plus-agarose beads (Calbiochem, San Diego, CA) for 60 min. Polyclonal anti-SR-BI antibody directed to the extracellular domain (1:60 dilution) (NOVUS, Biochemicals, NB-400-113) or an equal amount of control rabbit IgG was added to the samples and incubated overnight at 4°C with agitation. Protein A/G Plus-agarose beads were then added in the reaction mixture to allow binding of SR-BI antibody or control IgG to the beads for 2h at 4°C. The beads were washed three times with TE buffer (10 mM Tris pH 7.5 and 5 mM EDTA) containing 150 mM NaCl and the bound proteins were eluted by boiling for 3min in SDS sample buffer. Eluted proteins were separated by SDS-PAGE, and immunoblot analyses were performed.

Carotid Artery Reendothelialization: Carotid artery reendothelialization was studied following perivascular electric injury in 12-16 week-old male PDZK1+/– and PDZK1−/− littermates. 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 prior to the procedure. In additional experiments reendothelialization was compared in PDZK1−/− mice in which liver directed gene transfer of PDZK1 was performed by intravenous administration of control lacZ-containing or murine PDZK1-containing adenovirus (1X10^{11} particles per mouse, Viraquest) 1d prior to artery
injury. A similar strategy has been used to selectively rescue SR-BI expression in liver. Five days following injury total cholesterol levels were measured, animals were injected with 5% Evans blue dye (Sigma-Aldrich), liver and carotid 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). All animal experiments were approved by the Institutional Animal Care and Utilization Committee at UT Southwestern.

References


7. Mineo C, Gormley AK, Yuhanna IS, Osborne-Lawrence S, Gibson LL, Hahner L, Shohet RV, Black S, Salmon JE, Samols D, Karp DR, Thomas


