Endothelial and Antithrombotic Actions of HDL

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Abstract—It is well recognized that high-density lipoprotein (HDL)-cholesterol is antiatherogenic and serves a role in mediating cholesterol efflux from cells. However, HDL has multiple additional endothelial and antithrombotic actions that may also afford cardiovascular protection. HDL promotes the production of the atheroprotective signaling molecule nitric oxide (NO) by upregulating endothelial NO synthase (eNOS) expression, by maintaining the lipid environment in caveolae where eNOS is colocalized with partner signaling molecules, and by stimulating eNOS as a result of kinase cascade activation by the high-affinity HDL receptor scavenger receptor class B type I (SR-BI). HDL also protects endothelial cells from apoptosis and promotes their growth and their migration via SR-BI–initiated signaling. As importantly, there is evidence of a variety of mechanisms by which HDL is antithrombotic and thereby protective against arterial and venous thrombosis, including through the activation of prostacyclin synthesis. The antithrombotic properties may also be related to the abilities of HDL to attenuate the expression of tissue factor and selectins, to downregulate thrombin generation via the protein C pathway, and to directly and indirectly blunt platelet activation. Thus, in addition to its cholesterol-transporting properties, HDL favorably regulates endothelial cell phenotype and reduces the risk of thrombosis. With further investigation and resulting greater depth of understanding, these mechanisms may be harnessed to provide new prophylactic and therapeutic strategies to combat atherosclerosis and thrombotic disorders. (Circ Res. 2006;98:1352-1364.)

Key Words: atherosclerosis ■ endothelial nitric oxide synthase ■ endothelium ■ HDL ■ nitric oxide ■ prostacyclin ■ protein C ■ thrombin ■ thrombosis

The risk of atherosclerosis is inversely related to circulating levels of high-density lipoprotein cholesterol (HDL-C), and the Framingham Heart Study demonstrated that the association is independent of low-density lipoprotein (LDL) cholesterol. In addition, clinical trials with agents that increase HDL show that elevations in the lipoprotein decrease the incidence of cardiovascular events. Furthermore, there is evidence that the risk for restenosis following a vascular intervention is inversely related to HDL. HDL classically functions in reverse cholesterol transport (RCT), removing cholesterol from peripheral tissues and delivering it to the liver and to steroidogenic organs by binding of the major HDL apolipoprotein apolipoprotein A-I (apoA-I) to the high-affinity HDL receptor scavenger receptor class B type I (SR-BI). In mouse models of atherosclerosis, both apoA-I and SR-BI provide atheroprotection, and the provision of apoA-I or HDL also attenuates neointima formation after artery injury in the context of experimental hypercholesterolemia. The protective nature of HDL has been previously attributed to its role in RCT. However, the basis for...
HDL-related atheroprotection remains complex and not fully understood, and alternative mechanisms of action of HDL must be considered.

Our understanding of the role of apoA-I/HDL in cardiovascular health and disease has entered a new era recently with the discovery that HDL has direct actions on the vascular endothelium and on mechanisms mediating thrombosis. This review highlights recent advances in this realm of HDL biology by summarizing findings from experimentation in cell culture and in animal models and from observational and interventional studies in humans. In particular, the capacity of HDL to modify endothelial NO synthase (eNOS) expression and activity and the production of the atheroprotective signaling molecule nitric oxide (NO) are discussed (Figure 1).

HDL Modulation of eNOS

eNOS, NO, and Atherosclerosis

During the early stages of hypercholesterolemia-induced vascular disease, there is a dramatic decrease in bioavailable endothelium-derived NO, which is a potent vasodilator with multiple additional effects on endothelium, vascular smooth muscle, and events at the luminal surface. NO is generated by eNOS on the conversion of L-arginine to L-citrulline, and eNOS activity is modulated by agonists of diverse cell surface receptors and by physical stimuli such as hemodynamic shear stress. In the initial phase of hypercholesterolemia, there is exclusively impaired responsiveness to receptor-dependent stimuli of eNOS such as acetylcholine (Ach), whereas responsiveness to receptor-independent stimuli such as the calcium ionophore A23187 is not altered. As atherosclerotic disease progresses, there is nonspecific inhibition of NO bioavailability that is at least partly attributable to enhanced inactivation of NO by superoxide ($O_2^-$) anions. These processes result in increased neutrophil adherence to the endothelium and comprise key components of the pathogenesis of atherosclerosis. NO deficiency also enhances smooth muscle cell proliferation and platelet aggregation and adhesion. In vivo evidence of these mechanisms includes studies in rabbit models of hypercholesterolemia in which the chronic inhibition of NO synthesis causes marked acceleration of the development of vascular dysfunction and intimal lesions. In addition, mice lacking apolipoprotein E (apoE), which develop spontaneous atherosclerosis and display attenuated NO-mediated vasodilation, have more rapid progression of atherosclerosis when subjected to either long-term NOS antagonism or genetic eNOS deficiency. Thus, multiple lines of investigation indicate that NO is atheroprotective and that NO deficiency is critically involved in the pathogenesis of hypercholesterolemia-induced vascular disease.
Impact of HDL on eNOS Localization
Because eNOS activity is acutely regulated by multiple extracellular stimuli and the NO produced is a labile, cytotoxic messenger molecule with primarily paracrine function,30,31 the intracellular site of NO synthesis has a major influence on the biological impact of the enzyme. In cultured endothelial cells, eNOS is primarily associated with caveolae.32,33 Caveolae are specialized, lipid-ordered plasma membrane microdomains enriched in cholesterol, glycosphingolipids, sphingomyelin, and lipid-anchored membrane proteins, and they contain a variety of signal-transduction molecules. Both N-terminal myristoylation and palmitoylation of eNOS are necessary for optimal targeting to caveolae.34 In addition to co- and posttranslational modifications of resident proteins, membrane cholesterol is essential for normal caveolae function.35 Oxidized LDL (OxLDL) causes depletion of caveolae cholesterol in endothelial cells via the scavenger receptor CD36, leading to eNOS redistribution to an intracellular locale and an attenuated capacity to activate the enzyme.36,37 In parallel, wild-type mice with normal eNOS localization in caveolae have a decline in blood pressure in response to Ach administration, whereas hypercholesterolemic apoE-null mice display no change in blood pressure with Ach, and eNOS is not present in caveolae. However, normal eNOS localization and Ach response are apparent in apoE/CD36 double-knockout mice.38 These findings indicate that pathological lipoprotein and cholesterol status disrupts normal eNOS subcellular localization and...
HDL levels and the risk for atherosclerosis and HDL are complex, and they may vary significantly.

The early effects of OxLDL on eNOS localization and activity can be either positive or negative. Additionally, there is a strong negative correlation between HDL levels and the risk for atherosclerosis and HDL mediates cholesterol trafficking. The impact of HDL on the early effects of OxLDL on eNOS localization and activation in caveolae has been determined. The addition of HDL to cell culture medium containing OxLDL prevents eNOS displacement from caveolae, and it additionally restores Ach-induced stimulation of the enzyme. Furthermore, the OxLDL-induced fall in caveolae sterol content is prevented by cotreatment with HDL. The ability of HDL to maintain the concentration of caveolae-associated cholesterol in the face of OxLDL is not related to the inhibition of cholesterol removal from caveolae by OxLDL; instead, it is attributable to the provision of cholesterol esters by HDL (Figure 1, mechanism no. 1). Moreover, SR-BI is expressed in endothelial cells and is highly enriched in endothelial cell caveolae, and the receptor mediates the ability of HDL to reverse the impact of OxLDL on eNOS localization and function. Thus, in the presence of OxLDL, one of the actions of HDL that occurs through SR-BI is to preserve the unique lipid environment within caveolae, thereby maintaining the normal subcellular localization and function of eNOS.

In additional studies, it has been shown that the apoA-I mimetics L-4F and D-4F protect endothelial cell function by preventing LDL from uncoupling eNOS activity to favor O$_2^-$ anion production over NO production (Figure 1, mechanism no. 2). These processes involving modification of the impact of LDL and OxLDL on the endothelium may explain at least a portion of the antiatherogenic properties of HDL.

**HDL Activation of eNOS**

In addition to the capacity to preserve eNOS localization when the lipid environment within caveolae is overly perturbed and to prevent uncoupling of the enzyme, studies of cultured endothelium have demonstrated that HDL is a potent agonist of eNOS (Figure 1, mechanism no. 3). Heterologous expression experiments in Chinese hamster ovary cells further revealed that SR-BI mediates the activation of eNOS by HDL. Similarly, HDL enhances endothelial- and NO-dependent relaxation of aortic rings from wild-type but not antioxidant-null mice. The functional linkage of SR-BI and eNOS is also demonstrable in isolated endothelial cell caveolae, revealing that all of the molecular machinery required for HDL-induced eNOS stimulation is associated with caveolae.

In both human and animal studies, apoA-I is the apolipoprotein principally responsible for the atheroprotective features of HDL. In cultured endothelial cells, potential apoA-I–eNOS interaction and perinuclear colocalization have been reported. However, eNOS enzymatic activation has not been observed with lipid-free apoA-I. In isolated endothelial cell plasma membranes, anti–apoA-I antibody blocks eNOS activation by HDL, whereas anti–apoA-II antibody causes enhanced eNOS stimulation by HDL. Thus, apoA-I is necessary but not sufficient for eNOS stimulation.

eNOS activity is regulated by complex signal transduction pathways that include the activation of kinases that alter the phosphorylation of the enzyme. Akt kinase activates eNOS

by directly phosphorylating the enzyme at Ser1179. Akt itself is phosphorylated and activated by tyrosine kinase (TK), which in turn is activated by a tyrosine kinase (TK). Both receptor TK and nonreceptor TK are involved in PI3-kinase/Akt-mediated eNOS activation by various agonists. In contrast to Ser1179, the phosphorylation of Thr497 of eNOS attenuates enzyme activity under certain conditions. eNOS is also modulated by mitogen-activated protein (MAP) kinases, and unlike Akt, the effect of MAP kinases on eNOS activity can be either positive or negative. Additionally, there is a critical role for calcium (Ca$^{2+}$)–calmodulin binding to eNOS in the regulation of enzyme activity. The requirements for these mechanisms in HDL activation of eNOS have been investigated. In bovine aortic endothelial cells, HDL causes eNOS phosphorylation at Ser1179, and parallel findings are obtained when SR-BI and eNOS are transfected into COS-M6 cells, which are a monkey kidney epithelial cell line lacking endogenous SR-BI or eNOS. In addition, dominant-
negative Akt inhibits both HDL-mediated phosphorylation and activation of the enzyme. In contrast, eNOS phosphorylation at Thr497 is not affected by HDL. PI3-kinase inhibition or dominant-negative PI3-kinase also blocks the Ser1179 phosphorylation and activation of eNOS by HDL. Further studies showed that the nonreceptor tyrosine kinase Src is an upstream stimulator of the PI3-kinase–Akt pathway in the HDL signaling pathway. Furthermore, HDL activates MAP kinase through PI3-kinase, and MAP kinase/extracellular signal-regulated kinase inhibition fully attenuates eNOS enzyme activation by HDL without affecting Akt or eNOS Ser1179 phosphorylation. Conversely, dominant-negative Akt does not alter HDL-induced MAP kinase activation. Thus, HDL stimulates eNOS through Src activation, which leads to parallel activation of Akt and MAP kinases, and it is the convergence of their actions that is required to activate the enzyme (Figure 1, mechanism no. 3).

Although not observed consistently in all reports, studies have indicated that HDL causes an increase in intracellular Ca\(^{2+}\) in endothelial cells. Use of Ca\(^{2+}\) chelation has further shown that Ca\(^{2+}\) is required for NO formation in response to HDL. Both LDL and HDL cause increases in endothelial cell intracellular Ca\(^{2+}\), which are attributable to Ca\(^{2+}\) release from internal stores via the activation of phospholipase C. Thus, the 2 lipoproteins impact calcium homeostasis via similar signaling pathways, and changes in intracellular Ca\(^{2+}\) are not the basis of the eNOS activation mechanism that is unique to HDL compared with LDL. The Ca\(^{2+}\) responses to HDL and LDL are sensitive to pertussis toxin, indicating dependence on G protein activation. Both native HDL and the lipids obtained from a chloroform:methanol extract of HDL activate increases in intracellular Ca\(^{2+}\), whereas apoA-I in phosphatidylcholine liposomes does not, suggesting the involvement of a lipid moiety. In studies limited to Chinese hamster ovary cells expressing SR-BI and eNOS, increases in intracellular ceramide levels have been demonstrated, with exogenous ceramide and HDL causing comparable eNOS activation. Thus, observations in a variety of model systems indicate that multiple signaling events are activated in endothelial cells by HDL, which likely converge to promote NO production by eNOS.

Role of HDL-Associated Molecules

The involvement of lysophospholipids associated with HDL in eNOS activation has been suggested. Sphingosylphosphorylcholine (SPC), sphingosine-1-phosphate (S1P), and lyso-sulfatide (LSF) are all potential cargos of HDL, SPC, S1P, and LSF cause parallel eNOS-dependent relaxation of precontracted aortic rings from mice. Experiments using aortas from mice lacking the lysosphospholipid receptor S1P\(_1\) indicate that 50% to 60% of the response to native HDL is mediated by lysosphospholipids in that model system (Figure 1, mechanism no. 3). However, because intravenous administration of HDL stimulates myocardial perfusion in vivo equivalently in wild-type and S1P\(_1\)-null mice but not in eNOS null mice, the role of eNOS in HDL-induced vascular responses remains clear, whereas the involvement of lysosphospholipids as physiological HDL-associated agonists may vary between endothelium from different vascular sources.

An additional HDL-associated molecule of potential interest is estradiol. It has been reported that HDL isolated from female humans or mice has greater capacity to stimulate eNOS than does HDL from male humans or mice. However, HDL-mediated responses occur at levels of the lipoprotein that would provide estradiol at concentrations as low as 5x10\(^{-16}\) mol/L, which is well below the levels known to activate eNOS directly. In addition, human studies showing that brief elevations in HDL enhance endothelial function have been done primarily in males (see below), and in other reports, differences in eNOS responses to male versus female HDL were not observed. Thus the role of estradiol in HDL activation of eNOS is currently unclear.

Impact of HDL on eNOS Protein Abundance

In addition to the regulation of NO production by signaling events that modulate eNOS enzymatic activity, important control of eNOS involves changes in the abundance of the enzyme. In cultured human endothelial cells, eNOS protein expression is increased 3-fold by HDL exposure for 24 hours, and the increase in eNOS protein is not associated with a rise in steady-state mRNA levels. Alternatively, it is related to a comparable 3-fold increase in the half-life of the protein, and this is mediated by PI3-kinase–Akt kinase and MAP kinase (Figure 1, mechanism no. 4). Thus, the same signaling mechanisms that underlie the acute activation of eNOS by HDL are evidently operative in upregulating the abundance of the enzyme as well.

HDL Modulation of eNOS in Humans

The relationship between HDL and endothelium-dependent vasodilation has been known since 1994, when it was reported that patients with elevated HDL have greater vasodilator and attenuated vasoconstrictor responses. Subsequent studies of flow-mediated vasodilation of the brachial artery showed that HDL-C is an independent predictor of endothelial function. In an interventional study of HDL elevation by niacin treatment for 3 months in patients with coronary artery disease and depressed HDL levels, niacin administration and the resulting 25% increase in HDL was associated with a marked improvement in flow-mediated vasodilation. The direct, short-term impact of HDL on endothelial function has also been investigated in humans. One study analyzed forearm blood flow responses in ATP-binding cassette transporter 1 (ABCA1) heterozygotes. Compared with controls, ABCA-1 heterozygotes (6 men and 3 women) had HDL levels that were decreased by 60%, and their blood flow responses to endothelium-dependent vasodilators were blunted in spite of them having normal endothelium-independent responses. Following a 4-hour infusion of apoA-I/phosphatidylcholine disks, HDL levels in the ABCA1 heterozygotes increased 3-fold and endothelium-dependent vasomotor responses were normalized. In addition, endothelial function is enhanced in hypercholesterolemic men with normal HDL levels shortly after the administration of apoA-I/phosphatidylcholine particles. These observations...
HDL Regulation of Endothelial Cell Apoptosis, Proliferation, and Migration

HDL and Endothelial Cell Apoptosis

Evidence has accumulated that endothelial cell apoptosis contributes to the pathogenesis of atherosclerosis. An intact endothelial cell monolayer plays a critical role in normal homeostasis in the vascular wall, and apoptosis of endothelial cells can occur on exposure to circulating factors and inflammatory cells, leading to disruption of endothelial monolayer integrity. In addition, disturbances in vascular function and even acute coronary events may be mediated by thrombogenic membrane “microparticles” released from apoptotic endothelial cells (see below). Multiple proatherogenic factors promote apoptosis in endothelium, and these include OxLDL, tumor necrosis factor-α (TNF-α), homocysteine, and angiotensin II. OxLDL causes a delayed but sustained increase in intracellular Ca\(^{2+}\) in endothelial cells, which results in cell death, and this effect is reversed by HDL and mediated by prevention of the sustained Ca\(^{2+}\) increase (Figure 2). The protection afforded by native HDL is mimicked by purified apoA-I, and it requires HDL binding to the cells and new protein synthesis. TNF-α−induced endothelial cell apoptosis is also inhibited by HDL, and this is associated with attenuated induction of CPP32-like protease (caspase 3), which is a component of all primary apoptotic pathways. Growth factor deprivation−related apoptosis of endothelial cells is also suppressed by HDL. This is attributable to blunting of the mitochondrial pathway of apoptosis, and the evidence includes HDL−induced attenuation of the dissipation of mitochondrial potential, the generation of oxygen−derived free radicals, the release of cytochrome c to the cytoplasm, and the activation of caspases 3 and 9. HDL also activates Akt and causes phosphorylation of the Akt target BAD, which favors BAD dissociation from BCL-X\(_{L}\), that is then free to inhibit mitochondria−mediated apoptosis. The HDL−associated lysophospholipids SPC and LSF protect endothelial cells from growth factor deprivation−related apoptosis via parallel mechanisms. In addition, the lysophospholipid S1P enhances endothelial cell survival with effects comparable to those of native HDL, and these responses are inhibited by knockdown of the S1P receptor EDG-1/S1P₁, by pertussis toxin, and by PI3-kinase and ERK pathway antagonists, suggesting that signaling by lysophospholipid components of HDL may be important for the inhibition of apoptosis. Studies of the apoA-I mimetic D-4F in a rat model of diabetes indicate that D-4F improves vascular reactivity and decreases endothelial cell fragmentation and sloughing, providing evidence that these mechanisms may be operative in vivo.

The potential role of SR-BI in the modulation of endothelial cell apoptosis has been recently addressed. Interestingly, heterologous expression experiments in Chinese hamster ovary cells demonstrated that SR-BI can induce a novel ligand−independent apoptotic pathway. Importantly, similar findings were obtained in comparisons of embryonic fibroblasts or aortic endothelial cells from wild-type versus SR-BI null mice. Further experiments showed that a putative redox motif CXXS residing at amino acids 323 to 326 of SR-BI is required for the promotion of apoptosis and that this is mediated by caspase 8, which is a component of the membrane−related apoptosis pathway. The proapoptotic effects of SR-BI were reversed by HDL and by eNOS, suggesting that under normal conditions, the antiapoptotic actions of NO prevail, whereas with low levels of HDL, SR-BI may promote endothelial cell death. The evidently complex mechanisms by which HDL and SR-BI regulate endothelial cell fate via impacts on different primary apoptotic pathways warrant further investigation, and the implications of these mechanisms in vivo remain to be determined.

HDL and Endothelial Cell Proliferation and Migration

The processes of endothelial cell proliferation and migration are crucial to both neovascularization and to a successful response to vascular injury. An intact endothelial cell monolayer modulates local hemostasis and thrombolysis and provides a nonpermeable barrier protecting vascular smooth muscle (VSM) 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 caused by disturbed shear stress, place the arterial wall at greater risk for vascular disease. Furthermore, whereas repeated endothelial removal worsens the severity of vascular lesions, enhanced reendothelialization blunts lesion formation. Providing another potential explanation for the cardiovascular protection afforded by HDL, it was demonstrated in the 1980s that the lipoprotein promotes endothelial cell proliferation. The proliferative response of endothelial cells to HDL is Ca\(^{2+}\)−dependent. In 1994, it was reported that HDL stimulates endothelial cell migration independent of cell proliferation. Responses to HDL and basic fibroblast growth factor (bFGF) were additive, and the former response was not sensitive to pertussis toxin or inhibition of phospholipase A2, whereas the latter was sensitive, indicating independent signaling mechanisms. In contrast, in later studies HDL activation of endothelial cell migration was prevented by pertussis toxin, mediated by the G protein−coupled S1P receptors EDG-1/S1P₁, and EDG-3/S1P₃, and affected by the S1P−rich and not the S1P−poor fractions of HDL. Dependence on PI3-kinase, p38 MAP kinase, and Rho kinase was also observed. Capillary tube formation stimulated by HDL in vitro is pertussis toxin sensitive but does not occur through p38 MAP kinase and alternatively requires p42/44 MAP kinase activity residing downstream of Ras. Recently, it has been shown that HDL stimulates endothelial cell migration in vitro in an NO−independent manner via 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, PI3-kinase, and p44/42 MAP kinases. Rapid initial stimulation of lamellipodia formation by HDL via SR-BI, Src kinases, and Rac also occurs. Paralleling the in vitro findings, carotid artery reendothelialization following perivascular electric injury is diminished in apoA-I−null mice, and recon-
HDL Regulation of Thrombosis

Clinical and Experimental Evidence of Antithrombotic Effects of HDL

As outlined above, the inverse correlation between HDL levels and the incidence of atherosclerotic cardiovascular disease is well documented in numerous studies. More specifically, arterial atherothrombosis is associated with dyslipidemia and dyslipoproteinemia. Detailed analysis of lipoprotein subclasses shows that patients with arterial atherothrombosis have lower plasma levels of large HDL particles, and an inverse relationship between the abundance of large HDL particles and the recurrence of adverse arterial events was recently described.

Venous thrombosis is a clinically distinct entity from arterial thrombosis, with notable differences in thrombus appearance, etiology, and appropriate therapeutic approaches. Whereas arterial thrombosis is highly dependent on platelet activation and altered blood flow, venous thrombosis is driven by processes involving coagulation factors and venous stasis. Clinical evidence linking dyslipidemia and dyslipoproteinemia with venous thrombosis has only recently emerged, and these studies indicate that elevated HDL is also associated with lower risk of venous thrombosis. Similar to arterial thrombosis, analysis of lipoprotein subclasses shows that greater abundance of large HDL particles is associated with lower risk of venous thrombosis. Moreover, asymptomatic atherosclerosis was associated with clinically symptomatic venous thrombosis in one report. Interestingly, observational studies revealed that statin use, which raises HDL levels, may decrease the occurrence of venous thrombosis by approximately 50% in postmenopausal women. However, because hormone replacement therapy causes elevations in HDL levels but results in increased risk of thrombosis and embolism, the mechanism by which HDL levels are raised should be considered when interpreting correlations between HDL levels and clinical events and whether they potentially represent direct or indirect causal relationships between elevated HDL and protection against thrombosis.

More concrete evidence of HDL-related anticoagulant and antiplatelet activity in humans includes the finding that the infusion of reconstituted HDL limits the development of a procoagulant state in healthy volunteers given low doses of endotoxin. These collective observations related to arterial and venous thrombosis and coagulation status in humans have heightened interest in the potential mechanisms by which HDL is antithrombotic.

The direct antithrombotic effects of HDL are more clearly demonstrated in animal models. In a rat model of acute arterial thrombosis, the infusion of apoA-I Milano caused a prolongation in the time of thrombus formation and a reduction in the weight of the thrombus, suggesting that HDL inhibits thrombus formation in vivo within a time frame relevant to acute thrombosis. The molecular mechanisms responsible for the antithrombotic effects of HDL are likely to be multiple, and they relate to the 3 categories of processes known as Virchow’s triad, which contribute to thrombus formation: (1) dysfunction of the cells within the vascular wall, particularly the endothelium; (2) disturbed blood flow; and (3) dysfunction of blood components (Figure 3). Many of the protective activities of HDL in the first 2 categories entail the NO-dependent ability of the lipoprotein to promote endothelial cell health and optimal vasoregulation, and they have been summarized above.

HDL and Prostacyclin Synthesis

In addition to promoting NO production, HDL causes enhanced prostacyclin synthesis, which can modify thrombosis as well as other intravascular events (Figure 3). Prostacyclin acts synergistically with NO to induce VSM relaxation, inhibit platelet activation, and diminish the release of growth factors that stimulate the local proliferation of VSM cells. Prostacyclin is synthesized from arachidonate derived from phospholipids of cellular membranes or from exogenous sources that include phospholipids and cholesteryl esters associated with circulating lipoproteins. The rate-limiting enzyme in prostacyclin production is cyclooxygenase (Cox), which exists as 2 isoforms. Cox-1 is constitutively expressed, and Cox-2 is inducible, and both isoforms promote prostacyclin synthesis in endothelial cells. The incubation of cultured endothelial cells with native HDL causes an increase in prostacyclin production, and delipidated HDL also enhances production but to a lesser extent than intact HDL, suggesting that both HDL-associated lipids and apolipoproteins are involved. The impact of HDL on prostacyclin production in endothelium occurs by both the provision of arachidonate and upregulation of Cox-2 expression.

Recently, it has been shown that HDL3 induces Cox-2 expression and prostacyclin release via a p38 MAP kinase/CREB-dependent pathway in endothelium that mimics the mechanism that occurs in VSM. Prostacyclin release increases when isolated rabbit and rat hearts are infused with HDL. There is minimal direct information on the effects of HDL on prostacyclin generation in humans, but it has been noted that plasma HDL-C levels correlate with the plasma concentration of the stable prostacyclin metabolite 6-keto PGF1α. Thus, an important potential antithrombotic property of HDL is its ability to promote the generation of prostacyclin.

HDL and the Mechanisms of Thrombus Initiation

During inflammation, cytokines induce the appearance of tissue factor and the selectins P-selectin and E-selectin on the surfaces of platelets and endothelial cells. These prothrombotic factors are also present on subsets of cell-derived, circulating microparticles. Such prothrombotic microparticles can contribute both to the initiation of thrombus formation when they bind to perturbed endothelium and to the progression of thrombus formation when they bind to the luminal
surface of a growing thrombus. Because the adhesive reactions between microparticles and endothelium or blood cells in a growing thrombus are critical to the thrombotic process, downregulation of the expression of selectins or of tissue factor inhibits thrombus formation. As summarized by Barter and colleagues, the phospholipid components of HDL contribute to the downregulation of E-selectin expression on endothelial cell surfaces. Furthermore, thrombin-induced endothelial cell tissue factor expression in vitro is directly downregulated by reconstituted HDL. Moreover, HDL may decrease tissue factor induction on endothelial cells indirectly by increasing the synthesis of NO. Consistent with the documentation of these processes in vitro, there is an inverse correlation between HDL levels and the abundance of P-selectin-positive platelets in humans.

In addition to the effects of HDL on tissue factor and selectin expression, HDL may also be antithrombotic because it prevents endothelial cell apoptosis. Along with being an important source of circulating microparticles, apoptotic endothelial cells enhance adhesive reactions between unactivated platelets and leukocytes and the intimal surface, and apoptotic endothelial cells exhibit procoagulant properties.

**HDL, Activated Protein C, and Coagulation Factors**

Thrombin generation via blood coagulation pathways induces platelet activation and platelet-derived growth factor release and directly causes fibrin clot formation by cleaving fibrinogen. The protein C pathway provides a major physiological anticoagulant mechanism to downregulate thrombin formation by inactivating factors Va and VIIIa in plasma, and the majority of currently identifiable genetic defects associated with venous thrombosis involve protein C pathway dysfunction. For example, the laboratory abnormality known as activated protein C (APC) resistance, which is caused by a subnormal anticoagulant response of patient plasma to APC, causes a mild hypercoagulable state and is a risk factor for venous thrombosis and ischemic stroke. There is a positive correlation between plasma apoA-I levels and anticoagulant response to APC/protein S in vitro, and there is also an inverse correlation between HDL and plasma thrombin activation markers such as prothrombin fragments F1+2 in vivo, suggesting that HDL may modify thrombin generation under normal conditions. HDL, particularly HDL2 or large HDL, functions as an anticoagulant cofactor when it enhances the inactivation of purified coagulation factor Va by APC and protein S in vitro, which blunts thrombin generation and fibrin clot formation. Moreover, studies of HDL infusions into cholesterol-fed rabbits indicate that HDL upregulates endothelial cell thrombomodulin, which is an additional anticoagulant factor that supports the generation of APC and the suppression of thrombin generation. Because thrombomodulin is both an antithrombotic factor via the generation of APC and an antiinflammatory factor via currently unknown mechanisms, the upregulation of thrombomodulin expression by HDL may have a variety of important implications regarding HDL action in humans.

**HDL Sphingolipids and Antithrombotic Activities**

HDL transports various sphingolipids that are present in plasma in the micromolar range, and at least 4 kinds of HDL sphingolipids may directly or indirectly contribute antithrombotic activity. First, glucosylceramide and related glycosphingolipids are anticoagulant lipid cofactors for APC anticoagulant actions, and low plasma levels of glucosylceramide are found in a significant number of venous thrombosis patients. Thus, HDL-bound glycosphingolipids may have clinical significance as antithrombotic lipid cofactors for APC and protein S. Second, sphingosine at concentrations similar to those found in plasma inhibits prothrombin activation on platelet surfaces and in model coagulation assay systems, and sphingosine appears to inhibit procoagulant interactions between factors Xa and Va. As such, sphingosine may directly downregulate thrombin generation.

Third, as mentioned above, various lysosphingolipids exert potent effects on cells via a family of G protein-coupled receptors, and HDL is the major plasma carrier of S1P, Because S1P and other lysosphingolipids contribute to HDL-related vasoactive and antiapoptotic activities and apoptosis of endothelial cells promotes thrombosis, the antiapoptotic activity of HDL mediated by both lysosphingolipids and NO may reduce the risk of thrombosis by reducing endothelial cell apoptosis. Fourth, HDL downregulates endothelial cell adhesion reactions, and at least some of this activity may be caused by lysosphingolipids. Because endothelial cell interactions with procoagulant, proinflammatory leukocytes and cell-derived microparticles involves adhesive reactions, HDL-associated lysosphingolipids may be antithrombotic by downregulating the expression of endothelial adhesive molecules.

**HDL and Fibrinolysis**

Fibrinolysis reactions provide proteolytic clearance of fibrin and the lysis of fibrin-rich thrombi by plasmin, which is formed following plasminogen activation. The contribution of hypofibrinolysis to arterial thrombosis has been described and is more evident than the contribution of hypofibrinolysis to venous thrombosis. HDL may promote fibrinolysis by downregulating plasminogen activator inhibitor-I (PAI-I) and by upregulating tissue plasminogen activator (t-PA). The oxidation of HDL alters the influence of HDL on fibrinolysis because oxidized HDL3, but not native HDL3, promotes PAI-I expression and consequently suppresses fibrinolysis.

**HDL and Platelets**

Platelet aggregation is inversely correlated with HDL levels in humans, suggesting that HDL has antiplatelet actions. The administration of reconstituted HDL to humans or the infusion of apoA-I Milano into rats inhibits platelet aggregation, further supporting the concept that HDL inhibits platelet activation in vivo. Mechanistically, although HDL may reduce platelet activation directly, HDL may also act indirectly on platelet activation via effects on endothelial cells. For example, HDL may regulate platelet function by downregulating the release of platelet activating factor or by upregulating NO synthesis and release from endothelial cells.
thromboxaneA2 (TxA2), and it upregulates prostacyclin production (see above),\textsuperscript{146,149} which can decrease platelet aggregation as well as blunt leukocyte-endothelial cell interactions and thereby prevent the initiation and progression of atherogenesis.\textsuperscript{143} Notably, HDL2 is more effective than HDL3 in promoting the antithrombotic effects of HDL involving the critical prostacyclin/TxA2 balance.\textsuperscript{144}

**Molecular Basis of HDL Signaling**

As outlined above, HDL induces a variety of signaling events that underlie the endothelial and antithrombotic actions of the lipoprotein.\textsuperscript{145,152} The molecular basis of HDL signaling has been investigated by interrogation of the proximal mechanisms in HDL activation of eNOS (Figure 4). In cultured endothelial cells, short-term exposure to HDL or methyl-\(\beta\)-cyclodextrin causes eNOS stimulation of similar magnitude, whereas cholesterol-loaded methyl-\(\beta\)-cyclodextrin does not. Cholesterol-free Lp2A-I particles comprised of lipid-free recombinant apoA-I and phosphatidylycholine also activate eNOS, whereas cholesterol-containing Lp2A-I particles do not. In addition, phosphatidylycholine-loaded HDL causes greater stimulation than native HDL, and blocking antibody to SR-BI, which retards cholesterol efflux, prevents eNOS activation. Furthermore, in a reconstitution system in COS-M6 cells, wild-type SR-BI mediates eNOS activation by both HDL and small unilamellar vesicles, whereas the SR-BI mutant AVI, which is incapable of efflux to small unilamellar vesicles, transmits signal only in response to HDL. Moreover, eNOS activation by both HDL and methyl-\(\beta\)-cyclodextrin is SR-BI dependent.\textsuperscript{153} These cumulative findings indicate that signal initiation by HDL requires cholesterol flux, that the apolipoprotein and phospholipid components of HDL are sufficient to initiate signaling, and that SR-BI serves as a cholesterol sensor on the plasma membrane (Figure 4, mechanism no. 1).

In the same model system, the features of SR-BI required for signal initiation were elucidated. Using SR-BII, which is a splice variant of SR-BI, and mutant and chimeric class B scavenger receptors, it was determined that the C-terminal cytoplasmic PDZ-interacting domain and the C-terminal transmembrane domain of SR-BI are both required for HDL signaling (Figure 4, mechanisms no. 2 and 3). In addition, a photoactivated derivative of cholesterol binds directly to the C-terminal transmembrane domain.\textsuperscript{153} Thus, key domains of SR-BI uniquely mediating HDL signaling have been identified.

**Current Questions**

There is presently great interest in the development of strategies to elevate HDL levels in humans to take advantage of the potent atheroprotective and antithrombotic properties of the lipoprotein in a prophylactic and/or therapeutic manner. This includes the administration of agents such as statins, fibrates, and nicotinic acid and apoA-I Milano/phospholipid complexes.\textsuperscript{154,155} In addition, apoA-I mimetic peptides, peroxisome proliferator-activated receptor (PPAR-\(\alpha\)) agonists, and cholesteryl ester transfer protein inhibitors are under consideration in clinical trials.\textsuperscript{155} As these efforts proceed to increase bioavailable HDL, the direct endothelial and antithrombotic actions of HDL deserve further investigation, and there are multiple current questions worthy of pursuit. This is particularly evident when one considers the complexities and limitations of extrapolating recent findings regarding HDL and vascular biology from tissue culture studies and animal models to the clinical setting. In addition, caution is warranted in the interpretation of associations found to date in patient populations because the HDL-related parameters and the functional or clinical outcomes being investigated may have been influenced or confounded by other factors that were difficult to assess in a controlled manner. As such, both laboratory and clinical investigations are needed to further clarify the mechanisms by which HDL has direct endothelial and antithrombotic actions.

First, questions remain regarding the molecular basis of HDL signal initiation. The role of primary constituents of the lipoprotein, as well as potential cargo molecules such as lysosphingolipids and estradiol, remains to be clarified. The molecular underpinnings of the requirements for the C-terminal transmembrane and cytoplasmic domains of SR-BI are also to be elucidated. For example, it remains unknown whether cholesterol binding to the C-terminal transmembrane domain plays a role in HDL/SR-BI signaling. The involvement of potential adapter molecules interacting with the C-terminal transmembrane domain such as PDZK1 is also yet to be assessed\textsuperscript{153} (Figure 4, mechanism no. 3). As importantly, the physiological and pathophysiological implications of these processes must be elucidated in vivo. For example, the relative importance of SR-BI in endothelium, as well as in the other varied cell types relevant to atherosclerosis and thrombosis, needs further clarification using cell-specific gain-of-function and loss-of-function strategies in vivo. In addition, the potential role of HDL/SR-BI in neovascularization should be tested. The recent discovery that HDL may protect against venous thrombosis merits further clinical assessment and mechanistic studies. The molecular basis for HDL-induced downregulation of thrombin generation via the protein C pathway and via other possible mechanisms warrants additional investigation. Laboratory studies focused on the chemical composition of various subclasses of HDL particles may pinpoint specific components that mediate antithrombotic activity and thus identify more specific analytes for diagnostic testing or for consideration as therapeutic agents in the context of both arterial and venous thrombosis. With greater knowledge of the basis for HDL modulation of endothelial cell function and thrombosis in hand, efforts to increase HDL levels can be complemented by enhancement of the ability of the lipoprotein to directly govern events in the vasculature. Through such a 2-pronged approach, the full potential of HDL to promote cardiovascular health and to inhibit thrombosis can ultimately be harnessed.

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Endothelial and Antithrombotic Actions of HDL

Mineo et al


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