Effects of HMG-CoA Reductase Inhibitors on Endothelial Function
Role of Microdomains and Oxidative Stress

R. Preston Mason, PhD; Mary F. Walter, PhD; Robert F. Jacob, PhD

Abstract—Certain pleiotropic activities reported for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are related to reductions in cellular cholesterol biosynthesis and isoprenoid levels. In endothelial cells, these metabolic changes contribute to favorable effects on nitric oxide (NO) bioavailability. Given the essential role of NO in preserving vascular structure and function, this effect of statins is of considerable therapeutic importance. Statins have been demonstrated to restore endothelial NO production by several mechanisms, including upregulating endothelial NO synthase (eNOS) protein expression and blocking formation of reactive oxygen species. In this article, we will discuss additional ways in which statins restore endothelial NO production and improve endothelial function. (1) Statins modulate membrane microdomain formation, resulting in reduced expression of proteins that specifically inhibit eNOS activation. (2) Statins reduce sterol biosynthesis, thus interfering with the formation of pathologic microdomains, including cholesterol crystalline structures. This observation has important implications for plaque stabilization, as these microdomains contribute to cholesterol crystal formation and endothelial apoptosis. Finally, (3) statins improve endothelial function by interfering with oxidative stress pathways through both enzymatic and nonenzymatic mechanisms. The relationships between membrane microdomains, cholesterol biosynthesis, and endothelial function will be discussed. (Circulation. 2004;109[suppl II]:II-34–II-41.)

Key Words: atherosclerosis ■ endothelial function ■ microdomain ■ nitric oxide ■ statins

Vascular Plasma Membranes Are Structurally Heterogeneous and Consist of Discrete Microdomains

The endothelial cell plasma membrane is composed of liquid-ordered microdomains assembled from lipid constituents; these have distinct biophysical characteristics and limited random movement.1–3 Such microdomains, or "lipid rafts," are typically detergent-resistant and highly enriched with cholesterol and sphingolipids, as compared with the overall membrane lipid bilayer.1,3 These regions of ordered lipid account for local sequestration of proteins that mediate signal transduction in a variety of cell types, including endothelial and vascular smooth muscle cells (SMCs). This new model of the membrane as a mosaic structure consisting of microdomains has displaced an earlier perspective on the membrane in which lipid and protein constituents diffuse in a random and unrestricted fashion. In certain disease conditions, such as hyperlipidemia, the composition and abundance of certain membrane microdomains are altered, contributing to mechanisms of atherogenesis in vascular cells (Figure 1).

A lipid raft subtype that has been the subject of intensive investigation is plasmalemmal caveolae. Caveolae are morphologically characterized as flask-shaped invaginations along the membrane surface (Figure 1). They are richly expressed in endothelial cells, fibroblasts, adipocytes, macrophages, and SMCs.4 In macrophages, caveolae have recently been shown to have a key role in cholesterol homeostasis, an important function during atherosclerosis.5 The principal component of caveolae is the protein caveolin, a scaffolding element that efficiently binds cholesterol and interacts with various signaling macromolecules, including G proteins and calcium-regulating proteins.4,6–8

Beyond its role in trafficking lipids between various intracellular compartments, caveolin also functions as a specific inhibitor of endothelial nitric oxide synthase (eNOS).8 By blocking access of eNOS to its cofactor, calcium/calmodulin, caveolin regulates the production of nitric oxide (NO) in the endothelium.9 In genetically engineered animals that specifically lack caveolin protein and caveolae microdomains, phenotypic changes included decreases in arterial dilation, myogenic tone, and contractility.10 Conversely, the expression of caveolin is markedly elevated under conditions of hypercholesterolemia, due to excessive cholesterol enrichment of the plasma membrane.11 High levels of caveolae are associated with a reduction in endothelial NO synthesis (Figure 1), which contributes to increased levels of superoxide and loss of NO-mediated benefits, including inhibition of platelet aggregation, SMC proliferation, and leukocyte adhesion.

From the Cardiovascular Division, Department of Medicine (R.P.M.), Brigham and Women’s Hospital, Harvard Medical School, Boston, Mass; and the Cardiovascular Research Group (R.P.M., M.F.W., R.F.J.), Elucida Research LLC, Beverly, Mass.

Correspondence to R. Preston Mason, PhD, 100 Cummings Center, Suite 135L, Beverly, MA 01915. E-mail rpmason@elucidaresearch.com

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Under normal conditions, eNOS is associated with cholesterol-enriched caveolae in endothelial cells, where its activity can be carefully regulated. However, in hyperlipidemia, lipoprotein particles modulate the activity and subcellular distribution of eNOS. Incubation of endothelial cells with low-density lipoprotein (LDL), particularly oxidized LDL (ox-LDL), causes an increase in binding of eNOS to CD36, which attenuates its activity and causes displacement of the protein from endothelial caveolae. In endothelial cells, CD36 and scavenger receptor BI (SR-BI) cofractionate with caveolae and also coimmunoprecipitate with caveolin-1. Ox-LDL also perturbs the levels of cholesterol associated with endothelial caveolae. In endothelial cells, CD36 and scavenger receptor BI (SR-BI) cofractionate with caveolae and also coimmunoprecipitate with caveolin-1. Ox-LDL also perturbs the levels of cholesterol associated with endothelial caveolae.

In addition to lipid rafts enriched in cholesterol and sphingolipid, a distinct type of membrane domain exists in vascular and macrophage foam cells that is causally related to cholesterol enrichment during atherosclerosis. This type of microdomain consists exclusively of free cholesterol and is prominent in cell membranes derived from atherosclerotic plaque. Direct evidence for the existence of such cholesterol microdomains in atherosclerotic cell membranes comes from our small-angle x-ray diffraction analyses, which have identified a highly ordered lipid structure with a molecular width of 34 Å (Figure 2). These membrane regions formed under conditions of hyperlipidemia consist exclusively of two cholesterol monohydrate molecules in a tail-to-tail orientation, based on the fact that a single cholesterol monohydrate crystal has a long dimension of 17 Å (Figure 3). We have also observed that oxidized derivatives of cholesterol form microdomains in a manner dependent on their 3-dimensional structure. By interfering with cholesterol biosynthesis, and reducing the relative concentration of cholesterol in the membrane, statins attenuate the formation of these crystalline-like membrane domains.

In normal cell plasma membranes, the ratio of cholesterol to phospholipid ranges between 0.5 and 1.0, under physiological conditions. The only mammalian cell type that contains cholesterol microdomains under physiological conditions is the fiber cell of the human ocular lens. The plasma membrane of the fiber cell contains molar ratios of
sterol to phospholipid as high as 3:1, resulting in cholesterol tail-to-tail bilayer structures similar to those observed in atherosclerotic vascular cell membranes and macrophage foam cells. Certain drugs that inhibit cholesterol biosynthesis have been shown to induce cataract development in animal models as a result of reducing essential levels of lens membrane cholesterol.

Cholesterol as an Important Determinant of Cell Membrane Structure and Function

Unesterified or free cholesterol is a major constituent of the vascular cell plasma membrane, where it regulates lipid bilayer dynamics and structure by modulating the packing of phospholipid molecules. The amount of free cholesterol in the cell is controlled by extracellular sources of cholesterol (serum LDL levels) and intracellular biosynthesis of sterols. Free cholesterol molecules partition into the plasma membrane where they assume an orientation with the long axis of the sterol lying parallel to the phospholipid acyl chains, increasing order in the upper acyl chain region of the membrane while decreasing constraints at the terminal methyl groups. In contrast, esterified cholesterol does not readily associate with the plasma membrane. By restricting the random motion of membrane lipids and the mean cross-sectional area occupied by neighboring phospholipid acyl chains, free cholesterol has a pronounced condensing effect on biologic membranes. Investigators have proposed that certain proteins localize preferentially to cholesterol-rich regions of the membrane, as the microenvironment created by cholesterol enrichment appears to be essential for protein folding and tertiary structure. It has also been hypothesized that proteins associated with areas enriched in cholesterol have specific roles in signal transduction, cell adhesion, motility, and the sorting and trafficking of membrane components.

In the membrane, cholesterol tends to coalesce into discrete clusters at cholesterol to phospholipid mole ratios in excess of 0.3 and to form separate domains at ratios in excess of 1.0. Theoretic and model membrane studies have demonstrated that the systematic addition of cholesterol to biologic membranes can eventually yield such sterol domains. At cholesterol levels of >50 mol % (compared with total phospholipid), an immiscible cholesterol monohydrate phase is formed with a reproducible unit-cell periodicity of 34 Å, in coexistence with the surrounding liquid crystalline lipid bilayer, consistent with that observed in atherosclerotic tissues. This measurement corresponds to a tail-to-tail arrangement of the sterol, as proposed in other model membrane studies using a variety of techniques.

Figure 2. Schematic diagram of changes in lipid raft structure and cell function during cholesterol enrichment and atherosclerosis. Subtypes of lipid rafts enriched with sphingolipid (blue) and cholesterol (red) include caveolae (1) that contain caveolin protein (green) and detergent-resistant membrane domains (2). The figure shows that with cholesterol enrichment, separate cholesterol crystalline membrane domains (3) form and precede the development of extracellular crystals (4). Cholesterol crystals contribute to mechanisms of cell injury and death, including apoptosis. With cholesterol enrichment, there is an increase in the number of caveolae, leading to inhibition of endothelial nitric oxide synthase (eNOS) activity along with the vascular benefits of nitric oxide. Loss of normal membrane structure and function with cholesterol enrichment is also associated with disruptions in calcium regulation and redox potential. Reprinted with permission from Mason RP et al.

Figure 3. A schematic model of cholesterol domain formation in atherosclerotic vascular cell membranes during hyperlipidemia. In the disease state, cholesterol microdomains with a molecular width of 34 Å are observed in the same plane as the overall lipid bilayer. Reprinted with permission from Mason RP et al.
physiological range to facilitate the normal activity of constituent proteins. When the amount of cholesterol exceeds these normal levels, there are broad and adverse consequences for vascular biology, leading to mechanisms of cell injury and death.

**Evidence for Cholesterol Crystalline Membrane Domains in Atherosclerotic Vascular Membranes**

Unstable atherosclerotic lesions are characterized by large extracellular lipid deposits consisting of both free and esterified cholesterol, phospholipid, and lesser amounts of triacylglycerol and fatty acids.\(^{57,58}\) Disruption in the integrity of the collagen-rich fibrous cap exposes elements of the blood to the thrombogenic lipid core, resulting in rapid thrombus formation.\(^{59}\) Free cholesterol in the plaque is associated with either membrane phospholipid or extracellular crystals and is a prominent feature of human and animal lesions.\(^{60,61}\) Although noncrystalline membrane cholesterol can readily exchange from the plaque with plasma lipoprotein particles, cholesterol in a crystalline state appears to be inert.\(^{57}\)

In mouse macrophage cells, formation of free cholesterol crystals is enhanced with an acyl-coenzyme A:acyltransferase inhibitor, as esterified cholesterol hydrolysis leads to free cholesterol accumulation.\(^{62}\) Microscopic cholesterol crystals form and extend out from the membrane with various morphologies that include plates, needles, and helices, as observed by scanning electron microscopy.\(^{16}\) Preventing crystal formation is an important goal, as cholesterol in this state does not respond well to pharmacological interventions that promote lesion regression.\(^{57,63}\) By modulating the chemical (eg, degree of acyl chain saturation and oxidation) and physical (eg, temperature) properties of the membrane, cholesterol crystalline domain formation may be slowed or blocked, thereby preventing subsequent extracellular crystal development. In models of atherosclerosis, systematic changes in the cholesterol content of vascular cell membranes have been measured and correlated with cholesterol microdomains.\(^{16}\) Under atherosclerotic-like conditions, prominent cholesterol domains with a unit-cell periodicity of 34 Å could be observed in SMC plasma membranes as free cholesterol levels in the membrane increased as a function of elevated serum cholesterol levels.\(^{16}\)

Recent findings indicate a role for membrane lipid peroxidation in endothelial dysfunction and membrane microdomain formation. Oxidation of LDL and cellular membranes is a free radical reaction by which molecular oxygen is incorporated into constituent polynsaturated fatty acids (PUFA) to yield lipid hydroperoxides. The first step in the reaction is the abstraction of a hydrogen atom from the bisallylic methylene group of PUFA to yield a lipid carbon radical. The lipid carbon radical can undergo resonance stabilization to form a radical with conjugated double bonds, which reacts with molecular oxygen to form lipid hydroperoxides.\(^{64}\) An elevation in reactive oxygen species (ROS) represents an essential component of atherosclerotic plaque development that contributes to nonenzymatic formation of isoprostanes from arachidonic acid.\(^{65-68}\) An important source of ROS are reduced nicotinamide adenine dinucleotide phosphate...
Effects of HMG-CoA Reductase Inhibitors on Cholesterol Microdomains, Endothelial Function, and Oxidative Stress

HMG-CoA reductase inhibitors (statins) provide therapeutic benefit by restoring normal endothelial NO synthesis under disease conditions (eg, hyperlipidemia). Beyond serum LDL reduction, statins enhance the release of NO through various mechanisms, such as increasing the expression of eNOS and inhibiting superoxide formation. Restoration of NO is essential, as the bioavailability of this molecule is dramatically reduced under conditions of hyperlipidemia, resulting in a loss in vasodilatory and cardioprotective benefits. Interest has focused on how changes in cellular lipids and oxidative stress levels can restore NO production by the endothelium. The release of NO promotes vasodilation while interfering with various atherogenic pathways, such as platelet adhesion, superoxide formation, expression of adhesion molecules, and SMC proliferation.71,72 Given the central role of endothelial NO production in preserving vascular function and inhibiting various proatherogenic pathways, the effects of statins on NO metabolism are of considerable importance.

Statins have been shown to improve NO synthesis by mechanisms unrelated to changes in serum LDL levels, including upregulation of eNOS expression73 and reduced superoxide formation.74,75 Additionally, statins stimulate endothelial NO production through a dramatic reduction in plasma membrane caveolin levels76 (Figure 1). By interfering with cholesterol biosynthesis and lowering plasma membrane cholesterol levels, atorvastatin was shown to attenuate the expression of caveolin-1 (Figure 5).76 Reduced caveolin-1 levels resulted in increased in NOS activation and NO production, in a highly dose-dependent fashion (Figure 6).76 These effects on NO metabolism were reversed with mevalonate. When incubated with increasing amounts of extracellular LDL, atorvastatin also promoted the agonist-induced association of eNOS and the chaperone Hsp90, resulting in potent eNOS activation.76 This finding indicates a novel effect of a statin on endothelial function by modulating plasma membrane microdomains and caveolin expression.

Statins may also enhance the normal production of endothelial NO by reducing membrane cholesterol levels, thereby restoring normal transport of L-arginine, the substrate for eNOS. Previous studies indicate that L-arginine uptake through the amino acid transporter in endothelial-cell plasma membranes may be impaired under conditions of atherosclerosis when membrane unesterified cholesterol levels are increased.49 Changes in the membrane microenvironment alter the active transport properties of membrane-bound proteins, such as cationic amino acid transporters. Thus, statins restore NO production by improving the uptake of this key amino acid as membrane cholesterol levels are returned to normal levels.

Under atherosclerotic conditions, higher ratios of cholesterol to phospholipid leads to the formation of discrete crystalline-like domains that alter the physicochemical properties of the membrane and serve as potential nucleating sites for extracellular crystals (Figure 1). Inhibition of endothelial-cell cholesterol biosynthesis reduces the amount of free cholesterol available for forming such domains. Statins may also interfere with cholesterol crystal development by reducing levels of unesterified cholesterol in the vascular cell plasma membrane.

Statins also reduce LDL oxidation, a key initiator of endothelial dysfunction. Statins interfere with LDL oxidation by several mechanisms: (1) blocking isoprenylation of rac 1, an important component of NADPH oxidase complex;74,77 (2) reducing expression of NADPH oxidase subunits;75 and (3) reducing serum levels of LDL available for oxidation. Additionally, atorvastatin was shown to reduce levels of a free radical initiator by enhancing catalase expression, independently of LDL changes.75 By reducing sources of oxidative stress, statins increase the bioavailability of NO by preserving eNOS activity and endothelial function while reducing the loss of NO through its reaction with superoxide.
Certain statins have demonstrated antioxidant effects by direct scavenging of free radical molecules, independently of their effects on lipid metabolism. A review of these studies, however, indicates that suprapharmacological (micromolar) concentrations of statin were required to produce this activity in these experimental models, and thus, the physiological relevance is uncertain. By contrast, we have observed that the active o-hydroxy metabolite of atorvastatin is capable of increasing the resistance of LDL to oxidative modification at pharmacological levels (nanomolar). This is a relevant observation, as the majority of atorvastatin in the plasma is in the form of active hydroxy metabolites. The dose-dependent effect of the o-hydroxy metabolite on human LDL oxidation at pharmacological concentrations in vitro is demonstrated in Figure 7. This distinct antioxidant mechanism is specifically attributed to the o-hydroxy moiety, as it is capable of quenching free radical reactions by proton-donation mechanisms after partitioning into the lipid environment. In support of this hypothesis, the activity of the atorvastatin active hydroxy metabolite could not be reproduced by the parent atorvastatin compound or other statins that lack this chemical feature, including lovastatin, pravastatin, rosuvastatin, and simvastatin. A similar protective effect of the atorvastatin metabolite against LDL oxidation was observed in other experimental models. As a further control, measurement of oxygen radical absorbance capacity for atorvastatin o-hydroxy metabolite was shown to be 10-fold greater than for Trolox, a water-soluble analog of vitamin E with well-characterized antioxidant properties.

**Conclusion**

Statins have an effect on endothelial function and NO production, independent of changes in serum LDL levels. By enhancing the expression of eNOS, interfering with oxidative stress pathways, and reducing expression of caveolin, statins enhance NO bioavailability. These pleiotropic activities of statins are related to reductions in cellular cholesterol biosynthesis and isoprenylation reactions. Additional effects beyond lipid metabolism for certain statins may be attributed to
distinct lipophilic properties and physicochemical properties. Collectively, these findings support a beneficial effect for statins on endothelial function that leads to improved vascular compliance while interfering with atherosclerotic pathways that lead to plaque vulnerability in coronary artery disease.

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