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## ROLE OF ENDOTHELIAL DYSFUNCTION IN ATHEROSCLEROSIS

JEAN DAVIGNON, MD; PETER GANZ, MD

**Abstract** —As the major regulator of vascular homeostasis, the endothelium exerts a number of vasoprotective effects, such as vasodilation, suppression of smooth muscle cell growth, and inhibition of inflammatory responses. Many of these effects are largely mediated by nitric oxide, the most potent endogenous vasodilator. Nitric oxide opposes the effects of endothelium-derived vasoconstrictors and inhibits oxidation of low-density lipoprotein. A defect in the production or activity of nitric oxide leads to endothelial dysfunction, signaled by impaired endothelium-dependent vasodilation. Accumulating evidence suggests that endothelial dysfunction is an early marker for atherosclerosis and can be detected before structural changes to the vessel wall are apparent on angiography or ultrasound. Many of the risk factors that predispose to atherosclerosis can also cause endothelial dysfunction, and the presence of multiple risk factors has been found to predict endothelial dysfunction. A number of clinical trials have shown that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) improve endothelial dysfunction in patients with coronary risk factors beyond what could be attributed to their impact on plasma lipids. Studies have elucidated several possible mechanisms by which statin therapy may improve endothelial dysfunction, including upregulation of nitric oxide production or activity and reduction of oxidative stress. (*Circulation*. 2004;109[suppl III]:III-27-III-32.)

**Key Words:** atherosclerosis ■ endothelial dysfunction ■ nitric oxide ■ statins

As the major regulator of vascular homeostasis, the endothelium maintains the balance between vasodilation and vasoconstriction, inhibition and stimulation of smooth muscle cell proliferation and migration, and thrombogenesis and fibrinolysis.<sup>1,2</sup> When this balance is upset, endothelial dysfunction occurs, causing damage to the arterial wall. Endothelial dysfunction is considered an early marker for atherosclerosis, preceding angiographic or ultrasonic evidence of atherosclerotic plaque.<sup>1</sup> The integral role of the endothelium in vascular health and of endothelial dysfunction in atherosclerosis has generated considerable interest in the potential for reversal of endothelial dysfunction with lipidlowering therapy.

### Regulatory Functions of the Endothelium

The normal, healthy endothelium regulates vascular tone and structure and exerts anticoagulant, antiplatelet, and fibrinolytic properties. The maintenance of vascular tone is accomplished by the release of numerous dilator and constrictor substances. A major vasodilative substance released by the endothelium is nitric oxide (NO), originally identified as endothelium-derived relaxing factor (EDRF). Other endothelium-derived vasodilators include prostacyclin and bradykinin.<sup>3</sup> Prostacyclin acts synergistically with NO to

inhibit platelet aggregation.<sup>1</sup> Bradykinin stimulates release of NO, prostacyclin, and endothelium-derived hyperpolarizing factor, another vasodilator, which contributes to inhibition of platelet aggregation.<sup>3</sup> Bradykinin also stimulates production of tissue plasminogen activator (t-PA), and thus may play an important role in fibrinolysis. The endothelium also produces vasoconstrictor substances, such as endothelin (the most potent endogenous vasoconstrictor identified to date) and angiotensin II. Angiotensin II not only acts as a vasoconstrictor but is also pro-oxidant<sup>4</sup> and stimulates production of endothelin. Endothelin and angiotensin II promote proliferation of smooth muscle cells and thereby contribute to the formation of plaque.<sup>3</sup> Activated macrophages and vascular smooth muscle cells, characteristic cellular components of atherosclerotic plaque, produce large amounts of endothelin.<sup>5</sup> Damage to the endothelium upsets the balance between vasoconstriction and vasodilation and initiates a number of events/processes that promote or exacerbate atherosclerosis; these include increased endothelial permeability, platelet aggregation, leukocyte adhesion, and generation of cytokines.<sup>6</sup> Decreased production or activity of NO, manifested as impaired vasodilation, may be one of the earliest signs of atherosclerosis.

## Nitric Oxide

Nitric oxide is a pivotal endothelium-derived substance. The hallmark of endothelial dysfunction is impaired endothelium-dependent vasodilation, which is mediated by NO. A defect in NO production or activity has been proposed as a major mechanism of endothelial dysfunction and a contributor to atherosclerosis.

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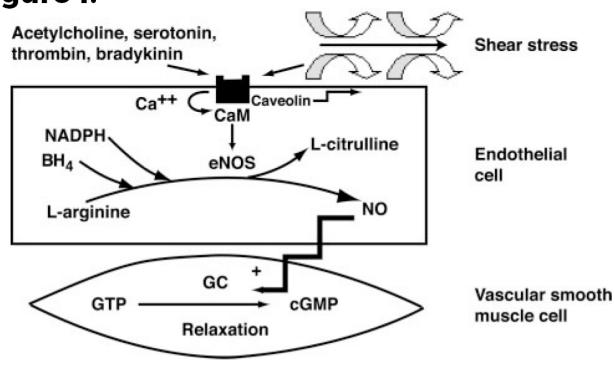
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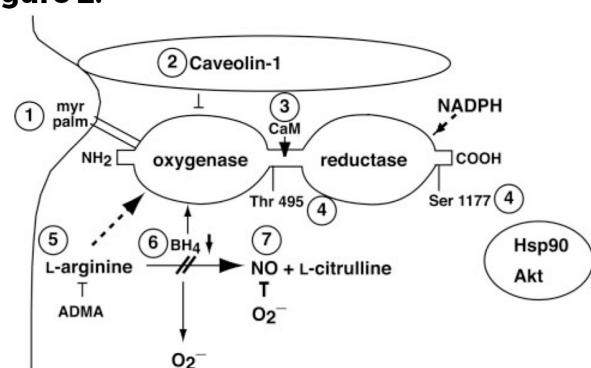
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**Figure 1.**



**Figure 2.**



**Figure 1.**

Production of nitric oxide (NO) by endothelial cells. NO is produced by the action of endothelial nitric oxide synthase (eNOS) on L-arginine. This reaction requires a number of cofactors, including tetrahydrobiopterin (BH<sub>4</sub>) and nicotinamide adenine dinucleotide phosphate (NADPH). Increased intercellular Ca in response to vasodilator agonists or shear stress displaces the inhibitor caveolin from calmodulin (CaM), activating eNOS. NO diffuses to vascular smooth muscle and causes relaxation by activating guanylate cyclase (GC), thereby increasing intracellular cyclic guanosine monophosphate (cGMP). Reprinted with permission from Behrendt D, Ganz P. Am J Cardiol. 2002;90(suppl):40L-48L.

**Figure 2.**

Endothelial nitric oxide synthase (eNOS) signaling. eNOS is composed of 2 globular protein modules (reductase and oxygenase domains) connected via a flexible protein strand. The reductase domain generates the electrons required for nitric oxide (NO) synthesis by binding reduced nicotinamide adenine dinucleotide phosphate (NADPH) and catalyzing its dehydrogenation. The electrons are transferred across the flexible protein strand to the oxygenase domain. This electron transfer is activated by calcium-dependent binding of calmodulin (CaM) to a specific binding site on the flexible protein strand. The oxygenase domain consists of the catalytic center responsible for NO production and binds heme, L-arginine, and tetrahydrobiopterin (BH<sub>4</sub>). The following steps are required for optimal NO production: (1) Localization of eNOS to caveolae (discrete microdomains of the plasma membrane) is necessary for efficient NO synthesis and requires cotranslational myristylation (myr) as well as posttranslational

palmitoylation (palm) of eNOS. (2) Caveolin-1, a major coat protein of caveolae, can interact with eNOS, resulting in eNOS inhibition. The interruption of this inhibitory interaction is required for eNOS activation. (3) CaM is an essential allosteric activator of eNOS. Binding of CaM to its specific binding site increases the rate of electron transfer from the reductase domain to the catalytic center of eNOS. (4) eNOS activity is regulated by phosphorylation at the serine residue 1177 (Ser 1177). This activating phosphorylation requires recruitment of the kinase Akt and of heat shock protein 90 (Hsp90). Hsp90 functions as a scaffold for eNOS and Akt. Phosphorylation at threonine 495 (Thr 495) inactivates eNOS. (5) The binding of the substrate L-arginine to the catalytic center of eNOS can be inhibited by the endogenous competitive antagonist asymmetric dimethyl arginine (ADMA). (6) BH4 is a required cofactor for NO synthesis. Depletion of BH4 may result in "uncoupling" of eNOS and production of superoxide ( $O_2^-$ ) instead of NO by eNOS. (7) Even when NO is produced properly, it can subsequently be rapidly inactivated by  $O_2^-$ , particularly under conditions of high oxidant stress.

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## Production of Nitric Oxide

Nitric oxide is formed in endothelial cells from its precursor L-arginine via the enzymatic action of endothelial NO synthase (eNOS), which is located in caveolæ (invaginations in cell membranes). The protein caveolin-1 binds to calmodulin to inhibit activity of eNOS; the binding of calcium to calmodulin displaces caveolin-1, activating eNOS and leading to production of NO. Cofactors such as tetrahydrobiopterin and nicotinamide adenine dinucleotide phosphate (NADPH) are also involved in NO production (Figure 1). The molecular basis of eNOS signaling is shown in greater detail in Figure 2.

Shear stress increases the expression of eNOS. Asymmetric dimethylarginine (ADMA) inhibits NO, and elevated levels of ADMA have been associated with endothelial dysfunction and atherosclerosis. The isoprenoid geranylgeranyl pyrophosphate, an intermediate factor in the cholesterol synthesis pathway, also inhibits synthesis of eNOS. Pharmacological inhibitors of eNOS include L-arginine analogues such as L-NG-monomethyl arginine (L-NMMA) and L-nitroarginine methylester (L-NAME).

## Functions of Nitric Oxide

Nitric oxide mediates endothelium-dependent vasodilation by opposing the effects of endothelium-derived vasoconstrictors such as angiotensin II and endothelin. It also inhibits platelet adherence and aggregation, leukocyte adhesion/infiltration, and proliferation of vascular smooth muscle cells. Nitric oxide prevents oxidative modification of low-density lipoprotein (LDL) cholesterol. Oxidation of LDL has been proposed as a major mechanism of the atherosclerotic process; furthermore, plasma and macrophage content of oxidized LDL in coronary plaques correlate with severity of acute coronary syndrome. Conversely, impaired production or activity of NO leads to events or actions that promote atherosclerosis, such as vasoconstriction, platelet aggregation, smooth muscle cell proliferation and migration, leukocyte adhesion, and oxidative stress. Oxidized LDL cholesterol increases synthesis of caveolin-1, which inhibits production of NO by inactivating eNOS. Oxidative stress can also interfere with the production and activity of NO by a number of mechanisms that are independent of LDL. For example, the free radical superoxide anion rapidly inactivates NO and destroys tetrahydrobiopterin, a cofactor required for NO synthesis.

## Clinical Assessment of Endothelial Function

Endothelial function can be assessed invasively using acetylcholine, which induces endothelium-dependent dilation and smooth muscle-mediated constriction. In healthy coronary arteries, endothelium-dependent dilation predominates. In the presence of endothelial damage, vasoconstriction predominates. The coronary artery diameter is compared by quantitative angiography before and after infusion of acetylcholine. The functional status of the coronary microvasculature can also be assessed using intracoronary Doppler ultrasound to measure blood flow in resistance vessels in response to substances that produce either endothelial-dependent or endothelial-independent vasodilation. Venous occlusion plethysmography has been used to measure vasomotor responses of forearm resistance vessels during infusion of acetylcholine into the brachial artery. Another noninvasive method of detecting endothelial dysfunction uses high-resolution ultrasound to measure the brachial artery diameter in response to reactive hyperemia. Reactive hyperemia induces increased blood flow and shear stress, stimulating NO release and flow-mediated dilation (FMD) that can be quantified as an index of vasomotor function. The systemic nature of atherosclerosis is reflected by the close correlation between endothelial dysfunction in the forearm and coronary endothelial dysfunction. These findings also suggest that noninvasive assessment of peripheral arteries may be useful for determining the effects of risk factors on endothelial function and for evaluating the effects of therapy. Finally, endothelial function correlates inversely with serum C-reactive protein (CRP).

## Endothelial Dysfunction and Atherosclerosis

A study by Ludmer et al using the acetylcholine test provided the first evidence in humans of impaired endothelium-dependent vasodilation in the presence of atherosclerosis. These investigators observed paradoxical constriction in the arteries of patients with mild coronary artery disease (CAD), as well as in those with advanced CAD, indicating that endothelial dysfunction is present in the early stage of atherosclerosis. Furthermore, in studies using either the acetylcholine test or measurement of FMD, endothelial dysfunction was detected at both the conduit and microvascular levels in patients with coronary risk factors but no angiographic or ultrasound evidence of structural CAD. These results confirm that endothelial dysfunction is present in the preclinical stage of atherosclerosis. Endothelial dysfunction of the microvasculature has also been associated with exercise-induced myocardial ischemia in patients without hemodynamically significant CAD of the epicardial arteries, suggesting that endothelial dysfunction of the microcirculation may contribute to ischemia when myocardial oxygen demand is increased.

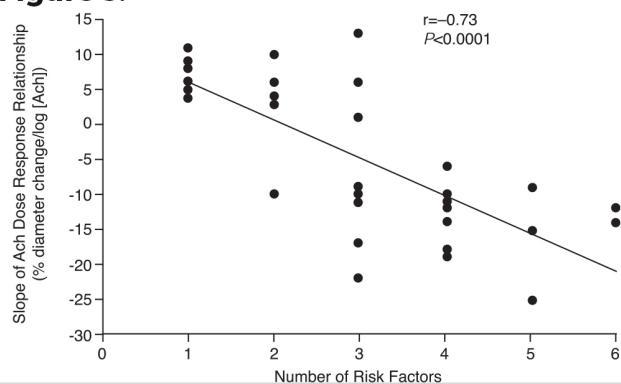
## Coronary Risk Factors Predict Endothelial Dysfunction

There has also been considerable interest in the links between endothelial dysfunction and coronary risk factors. Several studies have shown a correlation between endothelial dysfunction and the presence of coronary risk factors in persons with no clinical evidence of coronary disease. Many of the traditional coronary risk factors that predispose a person to the development of atherosclerosis, such as hypercholesterolemia, hypertension, smoking, diabetes, and a positive family history of premature CAD, are also associated with endothelial dysfunction.

The risk factor score (ie, the total number of risk factors in a given patient) has been found to be a potent independent predictor of endothelial dysfunction as measured by FMD20 and by the acetylcholine test. The total number of risk factors was significantly correlated with the response to acetylcholine (Figure 3).

Other proposed coronary risk factors, such as remnant lipoproteins, have also been shown to be associated with impaired endothelium-dependent vasodilation. Lipoprotein(a), an independent risk factor for coronary heart disease (CHD), stroke, and peripheral atherosclerosis, did not appear to be related to impaired endothelium-dependent vasodilation in a study by Schlaich et al. However, the production and release of basal NO was enhanced in subjects with elevated lipoprotein(a) levels, suggesting a compensatory response to as-yet-unidentified atherosclerotic effects of lipoprotein(a). In another study, small artery compliance was inversely correlated with lipoprotein(a) levels in patients with atherosclerosis. In these patients, increased lipoprotein(a) levels might cause endothelial dysfunction measurable by decreased small artery elasticity. Elastic properties of diabetic vessels were not directly related to lipoprotein(a) levels.

**Figure 3.**



**Figure 3.**

Relation between vasomotor response to acetylcholine (ACH) and the number of coronary risk factors. Reprinted with permission from Vita JA et al. Circulation. 1990;81:491-497.

## Endothelial Dysfunction Predicts Clinical Outcome

Research linking endothelial dysfunction with risk factors for coronary disease is intriguing and opens potentially useful avenues of investigation into the pathophysiology of coronary disease and also into the development of therapies targeting the endothelium. Given the links between endothelial dysfunction and coronary risk factors, it is not surprising that endothelial dysfunction is also associated with clinical events related to atherosclerosis. A study in patients with mild (nonobstructive) CAD found that severe coronary endothelial dysfunction significantly increased the risk of cardiac events over an average follow-up of 28 months. In contrast, patients with mild dysfunction or normal endothelial function experienced no cardiac events.<sup>28</sup> Evidence from subsequent studies reinforced the concept that coronary endothelial function may be a useful prognostic indicator. Coronary endothelial vasodilator dysfunction was found to independently predict progression of atherosclerosis and risk of cardiovascular events over a median follow-up of 7.7 years, even after the data were adjusted for conventional coronary risk factors. In the largest such study to date, epicardial and microvascular coronary endothelial dysfunction independently predicted acute cardiovascular events in patients with and without CAD.

In a study in which vasodilation was evaluated using plethysmography of forearm blood flow in response to acetylcholine (endothelium-dependent) and sodium nitroprusside (endothelium-independent), patients who experienced cardiovascular events over a mean follow-up of 4.5 years showed impaired vasodilator responses. Another study monitored 73 patients who underwent cardiac catheterization for chest pain and evaluation of brachial artery FMD by high-resolution ultrasound over a mean of 5 years. Cardiovascular events including percutaneous and surgical revascularization occurred more often in patients with impaired FMD (10%) compared with patients with preserved FMD (10%).

## Correction of Endothelial Dysfunction

A number of interventions have been shown to be effective in restoring endothelium-dependent vasodilation. These include lipid-lowering therapy (eg, 3-hydroxy-3-methylglutaryl coenzyme A [HMG-CoA] reductase inhibitors [statins], cholestyramine, or LDL apheresis), angiotensin-converting enzyme inhibitors, antioxidants, reducing hyperglycemia, diet, and exercise. The effectiveness of dietary L-arginine on endothelial dysfunction in humans is not fully established and further research is needed. The following discussion focuses primarily on the role of statins in improving endothelial dysfunction.

## Evidence for Beneficial Effects of Statins

As early as 1994, statin therapy was shown to improve endothelium-dependent dilation of coronary and peripheral arteries in patients with hypercholesterolemia. In one study, 6 months of treatment with pravastatin (10 to 20 mg/d) improved endothelium-dependent coronary vasomotion in patients with hypercholesterolemia. In another study of patients with atherosclerosis, cholesterol lowering with lovastatin (40 mg twice daily) significantly improved endothelium-mediated responses in the coronary arteries after 6 months.

However, improvement has been detected even earlier after initiation of therapy. For example, in hypercholesterolemic, postmenopausal women who received atorvastatin (10 mg/d) for 8 weeks, a significant increase in endothelium-dependent dilation as assessed by FMD of the brachial artery was evident after only 2 weeks, with further increases after 4 and 8 weeks. In hypercholesterolemic patients with perfusion abnormalities, 12 weeks of treatment with fluvastatin (40 to 80 mg/d) significantly increased myocardial perfusion in ischemic segments by 30% ( $P < 0.001$ ). In normal segments, perfusion increased by only 5% ( $P < 0.005$ ). Another study in patients with moderately elevated cholesterol levels showed that the vasodilator response to acetylcholine as determined by forearm blood flow was significantly ( $P < 0.005$ ) increased after 1 month of treatment with simvastatin 20 mg/d, and this improvement was further enhanced after 3 months. In the Reduction of Cholesterol in Ischemia and Function of the Endothelium (RECIFE) trial, 6 weeks of pravastatin therapy (40 mg/d) rapidly increased FMD compared with placebo in patients with acute coronary syndromes. Changes in FMD were not correlated with decreases in total and LDL cholesterol, suggesting that the improvement in endothelial function was not related to the lipidlowering effects of the statin. Augmented endotheliumdependent dilation has been noted in the forearm of healthy, normocholesterolemic men after only 1 day of high-dose atorvastatin (80 mg), even before appreciable reduction in plasma LDL cholesterol or CRP could be detected. This rapid increase in dilation is consistent with a cholesterolindependent effect of statins. Studies using positron emission tomography have shown that aggressive lipid-lowering therapy for 3 to 4 months reduces the size and severity of perfusion defects in patients with CAD and hypercholesterolemia, and this improvement was attributed to enhanced endothelial function. However, a study in patients with CAD and mildly elevated cholesterol levels found no difference at 6 months in coronary endothelial vasomotor function between placebo and simvastatin (40 mg/d) groups, although simvastatin therapy markedly improved the lipid profile. Endothelial function was assessed in both epicardial arteries (using acetylcholine and angiography) and the microvasculature (coronary blood flow response to substance P). The authors proposed several explanations for the discrepancy between their findings and those of other studies. For example, subjects in this study had lower baseline total and LDL cholesterol levels, less severe baseline atherosclerosis, and relatively mild baseline endothelial dysfunction compared with subjects in studies in which improved endothelial function was observed.

## Identifying the Mechanism

Improvement in endothelium-dependent vasodilation has been achieved with cholestyramine and LDL-apheresis, implicating LDL cholesterol reduction as an important mechanism. However, the clinical benefits observed with statin therapy in large controlled trials appear to exceed the benefits that would be expected from the observed reductions in cholesterol, suggesting that nonlipid effects (as part of their multiple effects referred to as pleiotropic effects), including improvement in endothelial dysfunction, may be involved. A number of mechanisms have been proposed for these effects. One important pathway appears to be the effects of statins on NO production via increased availability of eNOS. In one study in hypercholesterolemic patients, the significant improvement in endothelium-dependent vasodilation noted after statin therapy was blocked by administration of L-NMMA, an inhibitor of eNOS. This finding suggested that increased bioavailability of NO was the mechanism for the improved endothelial function.<sup>22</sup> In another study, short-term therapy with pravastatin improved forearm vasodilation in normocholesterolemic subjects with CAD. This improvement was likewise blocked by the coadministration of L-NMMA. Of note, the improvement in endothelial vasodilatory function was independent of the cholesterol-lowering effects of statin therapy. As observed previously, the rapid action of statins that precedes a reduction in serum cholesterol is also suggestive of a lipid-independent effect.

Elevated levels of native LDL decrease the bioavailability of endothelium-derived NO and downregulate endothelial eNOS. A recent study using endothelial cells from human umbilical veins showed that simvastatin prevented the downregulation of eNOS by native LDL. Elevated LDL reduces NO production in part by increasing the interaction between caveolin-1 and eNOS. An in vitro study has shown that atorvastatin reduces caveolin-1 expression in endothelial cells, inhibiting the interaction between caveolin-1 and eNOS and resulting in increased NO production. The statin-induced reduction in caveolin-1 expression was independent of the extracellular LDL-cholesterol level.<sup>47</sup> The statin also promoted the agonist-induced association of eNOS and the chaperone heat shock protein 90 (Hsp90), resulting in the potentiation of eNOS activation. Another proposed mechanism for the increase in eNOS expression with statin therapy is increased stability of eNOS messenger RNA, which would permit preservation of eNOS expression in the presence of oxidized LDL.

Activation of the Rho/Rho kinase pathway reduces the stability of eNOS mRNA, whereas inhibition of this pathway augments the stability of eNOS mRNA. Statins inhibit the activity of Rho/Rho signaling by blocking the generation of geranylgeranyl pyrophosphate, an effect that is cholesterol-independent.

Statin therapy also reduces circulating levels of the adhesion molecules P-selectin and intercellular adhesion molecule-1 (ICAM-1) in hypercholesterolemic subjects. The reduction in adhesion molecules was associated with an increase in levels of NO.<sup>50</sup> These findings suggest that statins may reduce platelet and leukocyte adhesion as well as improve endothelial cell function.

The serine/threonine protein kinase Akt (protein kinase B) mediates the activation of eNOS, resulting in increased production of NO. Statins have been found to activate Akt in endothelial cells, thereby enhancing the phosphorylation of the endogenous Akt substrate

eNOS and producing an increase in NO. Oxidative stress has been shown to contribute to endothelial dysfunction and the development of atherosclerosis. The use of antioxidant supplementation to improve endothelial function has support from some studies. For example, in one study, an LDL-lowering plus antioxidant regimen (lovastatin plus probucol) improved endothelium-dependent vasomotor response. In another study, probucol was found to be useful in preventing coronary restenosis, whereas antioxidant vitamins failed to exert any benefit. Antioxidant vitamins have not been found beneficial in several clinical trials including the recent Heart Protection Study. However, these findings should not be taken as the final word on the use of antioxidants in CHD patients.<sup>10</sup> Antioxidants are a diverse group of compounds, and in view of the central role played by oxidized LDL in endothelial dysfunction and atherogenesis, the antioxidant effects of statin therapy and probucol-like compounds are still of great therapeutic interest. Statins prevent the downregulation of eNOS by oxidized LDL. Finally, statins also appear to reduce the atherogenic potential of lipoproteins. Atorvastatin hydroxy metabolites inhibit oxidation of LDL, VLDL, and HDL.<sup>55</sup> Atorvastatin has also been shown to suppress the cellular uptake of oxidized LDL by monocytes, a key step in the development of atherosclerosis.

## Conclusions

Endothelium and its product nitric oxide are key regulators of vascular health. Reduced bioavailability of NO is involved in the initiation, progression and complications of atherosclerosis. Not surprisingly, deficiency of nitric oxide in coronary or peripheral arteries is predictive of future cardiovascular events. Basic mechanisms involved in endothelial dysfunction have been clarified and suggest a plethora of new therapeutic targets. In particular, studies investigating the nonlipid effects of statins on vascular function constitute a promising avenue of research.

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