# PLEIOTROPIC EFFECTS OF STATINS

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■ **Abstract** Statins are potent inhibitors of cholesterol biosynthesis. In clinical trials, statins are beneficial in the primary and secondary prevention of coronary heart disease. However, the overall benefits observed with statins appear to be greater than what might be expected from changes in lipid levels alone, suggesting effects beyond cholesterol lowering. Indeed, recent studies indicate that some of the cholesterol-independent or "pleiotropic" effects of statins involve improving endothelial function, enhancing the stability of atherosclerotic plaques, decreasing oxidative stress and inflammation, and inhibiting the thrombogenic response. Furthermore, statins have beneficial extrahepatic effects on the immune system, CNS, and bone. Many of these pleiotropic effects are mediated by inhibition of isoprenoids, which serve as lipid attachments for intracellular signaling molecules. In particular, inhibition of small GTP-binding proteins, Rho, Ras, and Rac, whose proper membrane localization and function are dependent on isoprenylation, may play an important role in mediating the pleiotropic effects of statins.

## INTRODUCTION

Cardiovascular disease, in particular coronary heart disease (CHD), is the principal cause of mortality in developed countries. Among the causes of cardiovascular disease, atherosclerosis is the underlying disorder in the majority of patients. Although the development of atherosclerosis is dependent on a complex interplay between many factors and processes (1), a clear association has been established between elevated levels of plasma cholesterol and increased atherosclerotic disease (2, 3). Indeed, several landmark clinical trials, such as the Scandinavian Simvastatin Survival Study (4S) (4), Cholesterol and Recurrent Events (CARE) (5), Long-term Intervention with Pravastatin in Ischemic Disease (LIPID) (6), West of Scotland Coronary Prevention Study (WOSCOPS) (7), Air Force/Texas

Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS) (8), Heart Protection Study (HPS) (9), and the Anglo-Scandinavian Cardiac Outcome Trial Lipid-lowering Arm (ASCOT-LLA) (10), have demonstrated the benefit of lipid lowering with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins for the primary and secondary prevention of CHD.

## PHARMACOKINETIC PROPERTIES OF STATINS

Cholesterol is an essential component of cell membranes and is the immediate precursor of steroid hormones and bile acids (11). However, in excessive amounts, cholesterol becomes an important risk factor for cardiovascular disease, as demonstrated in clinical trials from the Framingham Heart Study (3, 12) and the Multiple Risk Factor Intervention Trial (13, 14). Although dietary cholesterol can contribute to changes in serum cholesterol levels, more than two thirds of the body's cholesterol is synthesized in the liver. Therefore, inhibition of hepatic cholesterol biosynthesis has emerged as the target of choice for reducing serum cholesterol levels (15).

The rate-limiting enzyme in cholesterol biosynthesis in the liver is HMG-CoA reductase (11), which catalyzes the conversion of HMG-CoA to mevalonic acid (16). Inhibitors of HMG-CoA reductase, or statins, were originally identified as secondary metabolites of fungi (17). HMG-CoA reductase catalyses the rate-limiting step of cholesterol biosynthesis, a four-electron reductive deacylation of HMG-CoA to CoA and mevalonate. One of the first natural inhibitors of HMG-CoA reductase was mevastatin (compactin, ML-236B), which was isolated from *Penicillium citrinium* by A. Endo et al. in 1976 (18). In its active form, mevastatin resembles the cholesterol precursor, HMG-CoA. When mevastatin was initially administered to rats, it inhibited cholesterol biosynthesis with a $K_i$ of 1.4 nM. Unfortunately, it also caused unacceptable hepatocellular toxicity and further clinical development was discontinued. Subsequently, a more active fungal metabolite, mevinolin or lovastatin, was isolated from *Aspergillus terreus* by Hoffman and colleagues in 1979 (19, 20). Lovastatin differs from mevastatin in having a substituted methyl group. Compared to mevastatin, lovastatin was a more potent inhibitor of HMG-CoA reductase, with a $K_i$ of 0.6 nM, but did not cause hepatocellular toxicity when given to rats. Lovastatin, therefore, became the first of this class of cholesterol-lowering agents to be approved for clinical use in humans. Since then, several new statins, both natural and chemically modified, have become commercially available, including pravastatin, simvastatin, fluvastatin, atorvastatin, cerivastatin, and most recently, pitavastatin and rosuvastatin (21). Indeed, statins have emerged as one of the most effective class of agents for reducing serum cholesterol levels.

Statins work by reversibly inhibiting HMG-CoA reductase through side chains that bind to the enzyme's active site and block the substrate-product transition state of the enzyme (22). Thus, all statins share an HMG-like moiety and inhibit

the reductase by similar mechanism (Figure 1). Recently, the structure of the catalytic portion of human HMG-CoA reductase complexed with different statins was determined (22). The bulky, hydrophobic compounds of statins occupy the HMG-binding pocket and block access of the substrate HMG. The tight binding of statins is due to the large number of van der Waals interactions between statins and HMG-CoA reductase. The structurally diverse, rigid, hydrophobic groups of the different statins are accommodated in a shallow nonpolar groove that is present only when COOH-terminal residues of HMG-CoA reductase are disordered. There are subtle differences in the modes of binding between the various statins, with the synthetic compounds atorvastatin and rosuvastatin having the greatest number of bonding interactions with HMG-CoA reductase (22). Statins bind to mammalian HMG-CoA reductase at nanomolar concentrations, leading to effective displacement of the natural substrate, HMG-CoA, which binds at micromolar concentrations (23).

Oral administration of statins to rodents and dogs showed that these drugs are predominantly extracted by the liver and resulted in >30%–50% reduction in plasma total cholesterol levels and substantial decrease in urinary and plasma levels of mevalonic acid, the end product of the HMG-CoA reductase reaction. Similar reduction in cholesterol synthesis and decrease in circulating total and low-density lipoprotein (LDL)-containing cholesterol (LDL-C) by these agents have been subsequently confirmed in humans. Because hepatic LDL-C receptors are the major mechanism of LDL-C clearance from the circulation, the substantial declines in serum cholesterol levels are accompanied by an increase in hepatic LDL-C receptor activity. Statins, therefore, effectively reduce serum cholesterol levels by two separate mechanisms. They not only inhibit endogenous cholesterol biosynthesis via HMG-CoA reductase inhibition but also increase cholesterol clearance from the bloodstream via increases in LDL-C receptor.

The rank order of potency for HMG-CoA reductase inhibition among the second-generation statins is simvastatin > pravastatin > lovastatin $\cong$ mevastatin, with tissue $IC_{50}$ values of simvastatin and mevastatin being approximately 4 nM and 20 nM, respectively (24). The $IC_{50}$ values for these statins correspond to their relative potency for lowering serum cholesterol levels in vivo (i.e., simvastatin > lovastatin) (25). The newer third-generation synthetic statins, which include fluvastatin, cerivastatin, the penta-substituted pyrrole atorvastatin, pitavastatin (NK-104), and rosuvastatin, are much more potent than the mevastatin derivatives. These newer statins are active compounds, which share some physico-chemical properties with pravastatin, but have greater lipophilicity and half-life (26). Consequently, these statins, especially atorvastatin, pitavastatin, and rosuvastatin, appear to be quite effective in lowering serum cholesterol levels, perhaps, in part, owing to their ability to bind hepatic HMG-CoA reductase at higher affinity and inhibit the enzyme for a longer duration.

Because statins differ in their tissue permeability and metabolism, they possess different potencies for extrahepatic HMG-CoA reductase inhibition. These differences in tissue permeability and metabolism may account for some of the observed

differences in their peripheral side effects (27). Lipophilic statins, such as simvastatin, are considered more likely to enter endothelial cells by passive diffusion than hydrophilic statins, such as pravastatin and rosuvastatin, which are primarily targeted to the liver. However, lipophilicity does not entirely predict the ability of statins to exert extrahepatic effects in animal and human studies, and so other unidentified factors may play a role. It may be that there are specific mechanisms for hydrophilic statins to enter extrahapetic cells, such as endothelial cells. Such a mechanism is present in the liver, where the organic anion transporter (OATP-C) enables hydrophilic statins to enter hepatocytes (28).

Until recently, all cholesterol-independent or "pleiotropic" effects of statins were believed to be mediated by inhibition of mevalonate synthesis. However, statins can reportedly bind to a novel allosteric site within the ß2 integrin function-associated antigen-1 (LFA-1), independent of mevalonate production (29). LFA-1 belongs to the integrin family and plays an important role in leukocyte trafficking and in T cell activation. Random screening of chemical libraries identified the HMG-CoA reductase inhibitor, lovastatin, as an inhibitor of the LFA-1/intercellular adhesion molecule (ICAM)-1 interaction.

## STATINS AND ISOPRENYLATED PROTEINS

By inhibiting L-mevalonic acid synthesis, statins also prevent the synthesis of other important isoprenoid intermediates of the cholesterol biosynthetic pathway, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) (11) (Figure 2). These intermediates serve as important lipid attachments for the posttranslational modification of a variety of proteins, including the $\gamma$ subunit of heterotrimeric G-proteins; Heme-a; nuclear lamins; and small guanosine triphosphate (GTP)-binding protein Ras; and Ras-like proteins, such as Rho, Rab, Rac, Ral, or Rap (30). Thus, protein isoprenylation permits the covalent attachment, subcellular localization, and intracellular trafficking of membrane-associated proteins. Members of the Ras and Rho GTPase family are major substrates for post-translational modification by prenylation (30, 31). Both Ras and Rho are small GTP-binding proteins, which cycle between the inactive GDP-bound state and active GTP-bound state (Figure 3). In endothelial cells, Ras translocation from the cytoplasm to the plasma membrane is dependent on farnesylation, whereas Rho translocation is dependent on geranylgeranylation (32, 33). Statins inhibit both Ras and Rho isoprenylation, leading to the accumulation of inactive Ras and Rho in the cytoplasm.

Because Rho is major target of geranylgeranylation, inhibition of Rho and its downstream target, Rho-kinase, is a likely mechanism mediating some of the pleiotropic effects of statins on the vascular wall (34, 35). Each member of the Rho GTPase family, which consists of RhoA, Rac, and Cdc42, serves specific functions in terms of cell shape, motility, secretion, and proliferation, although overlapping functions between the members could be observed in overexpressed
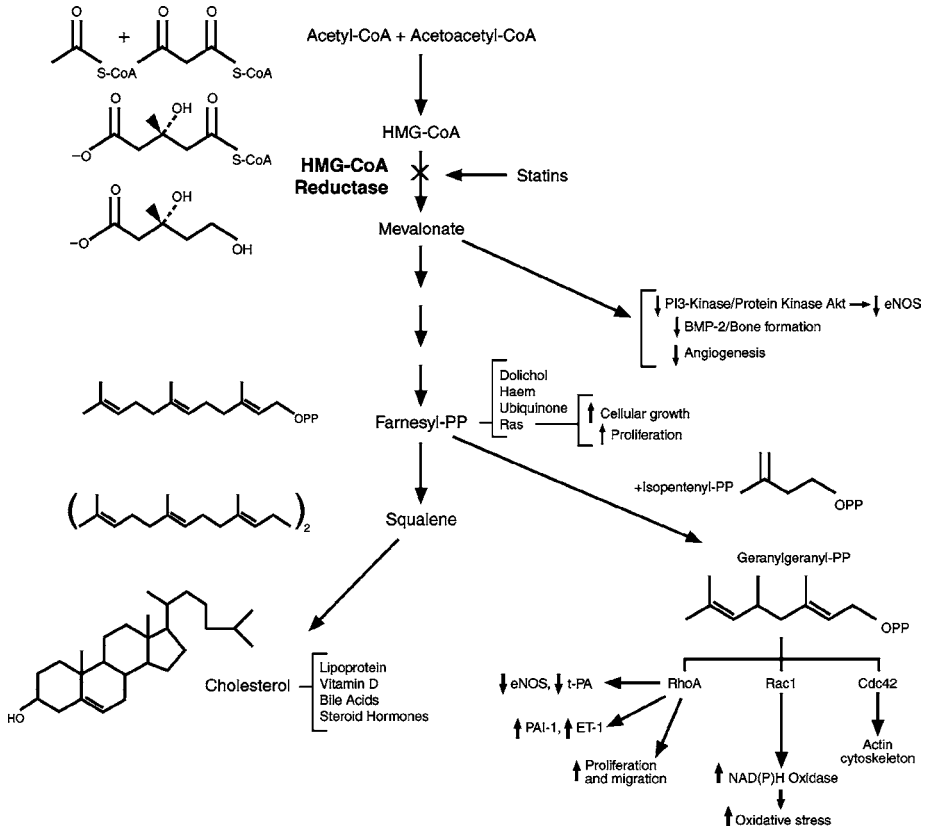
**Figure 2** Biological actions of isoprenoids. Diagram of cholesterol biosynthesis pathway showing the effects of inhibition of HMG-CoA reductase by statins. Decrease in isoprenylation of signaling molecules, such as Ras, Rho, and Rac, leads to modulation of various signaling pathways. BMP-2: bone morphogenetic protein-2; eNOS: endothelial nitric oxide synthase; t-PA: tissue-type plasminogen activator; ET-1: endothelin-1; PAI-1: plasminogen activator inhibitor-1.

systems. The activation of RhoA in Swiss 3T3 fibroblasts by extracellular ligands, such as platelet-derived lysophosphatidic acid, leads to myosin light chain phosphorylation and formation of focal adhesion complexes (30, 31, 36). Indeed, Rho-associated protein kinase increases the sensitivity of vascular smooth muscle to calcium in hypertension (37) and coronary spasm (38). In contrast, activation of Rac1 leads to the formation of lamellipodia and membrane ruffles, whereas activation of Cdc42 induces actin-rich surface protrusions called filopodia.

These distinct but complementary functions of Rho family members also extend to their effects on cell signaling. When cells undergo reorganization of their actin cytoskeleton in response to extracellular signals, such as growth factors, or
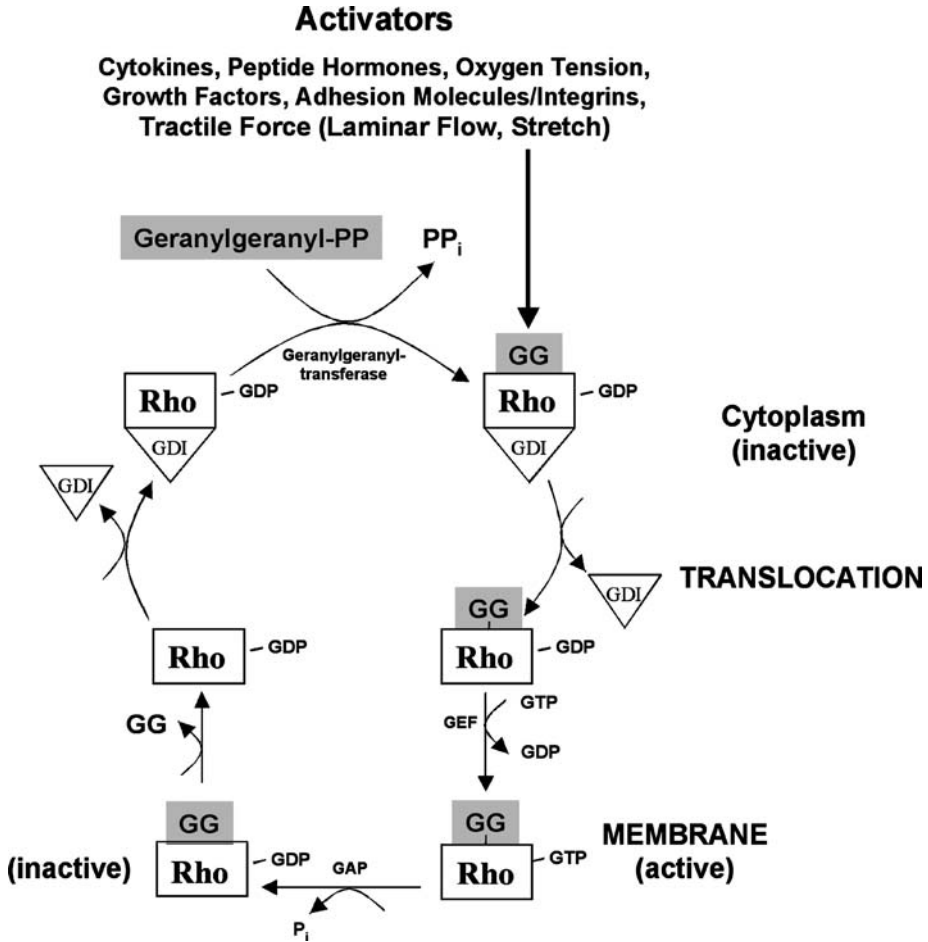
**Figure 3**   Regulation of Rho GTPase by isoprenylation. Rho proteins change between a cytosolic, inactive, GDP-bound state and an active, membrane, GTP-bound state. This cycle is controlled by several cofactors, including guanine nucleotide exchange factors (GEF), GTPase-activating proteins (GAP), and guanine nucleotide dissociation inhibitors (GDI). An important step in the activation of Rho GTPases is the posttranslational isoprenylation, which allows the translocation of Rho to the cell membrane and the subsequent activation.

during cell movement and mitosis, they alter the three-dimensional colocalization of intracellular proteins (30, 31). Thus, changes in Rho-induced actin cytoskeleton can affect intracellular transport, membrane trafficking, mRNA stability, and gene transcription. It is therefore not surprising to find that Rho-induced changes in the actin cytoskeleton and gene expression are related. Indeed, experimental evidence suggests that inhibition of Rho isoprenylation mediates many of the

cholesterol-independent effects of statins not only in vascular wall cells (32, 39), but also in leukocytes (40) and bone (41).

## STATINS AND ENDOTHELIAL FUNCTION

The vascular endothelium serves as an important autocrine and paracrine organ that regulates vascular wall contractile state and cellular composition. Hypercholesterolemia impairs endothelial function, and endothelial dysfunction is one of the earliest manifestations of atherosclerosis, occurring even in the absence of angiographic evidence of disease (42, 43). An important characteristic of endothelial dysfunction is the impaired synthesis, release, and activity of endothelial-derived nitric oxide (NO). Endothelial NO has been shown to inhibit several components of the atherogenic process. For example, endothelium-derived NO mediates vascular relaxation (44) and inhibits platelet aggregation (45), vascular smooth muscle proliferation (46), and endothelial-leukocyte interactions (47, 48). Inactivation of NO by superoxide anion ($O_2 \cdot ^-$) limits the bioavailability of NO and leads to nitrate tolerance, vasoconstriction, and hypertension (49, 50).

Acute plasma LDL-C apheresis improves endothelium-dependent vasodilatation (51), suggesting that statins could restore endothelial function, in part, by lowering serum cholesterol levels. However, in some studies with statins, restoration of endothelial function occurs before significant reduction in serum cholesterol levels (52–54), suggesting that there are additional effects on endothelial function beyond that of cholesterol reduction. Indeed, statins increase endothelial NO production by stimulating and upregulating endothelial NO synthase (eNOS) (32, 55). Furthermore, statins have been shown to restore eNOS activity in the presence of hypoxia (56) and oxidized LDL (ox-LDL-C) (32), conditions which lead to endothelial dysfunction. Statins also increase the expression of tissue-type plasminogen activator (t-PA) (57) and inhibit the expression of endothelin-1, a potent vasoconstrictor and mitogen (58). Statins, therefore, exert many favorable effects on the endothelium and attenuate endothelial dysfunction in the presence of atherosclerotic risk factors.

Although the effects of statins on Ras and Rho isoprenylation are reversed in the presence of FPP and GGPP, respectively, the effects of statins on eNOS expression is only reversed by GGPP and not by FPP or LDL-C (33). Indeed, direct inhibition of geranylgeranyltransferase or RhoA leads to increases in eNOS expression (33, 35, 59). These findings are consistent with a noncholesterol-lowering effect of statins and suggest that inhibition of RhoA by statins mediates the increase in eNOS expression. Indeed, statins upregulate eNOS expression by prolonging eNOS mRNA half-life but not eNOS gene transcription (33). Because hypoxia, ox-LDL-C, and cytokines such as TNF-$\alpha$ decrease eNOS expression by reducing eNOS mRNA stability, the ability of statins to prolong eNOS half-life may make them effective agents in counteracting conditions that downregulate eNOS expression.

Additional important effects of statin treatment on eNOS function include inhibition of caveolin (60, 61). Statins also increase eNOS activity via posttranslational activation of the phosphatidylinositol 3-kinase/protein kinase Akt (PI3K/Akt) pathway (55). Phosphorylation of Akt is an important event in several cellular activities. Indeed, production of NO by the endothelium can be regulated by phosphorylation and activation of eNOS by Akt, which is promoted in the presence of statins (62, 63). Caveolin-1 binds to eNOS in caveolae, thereby negatively regulating the enzyme (64). Exposure of cultured endothelial cells to hypercholesterolemic serum upregulates caveolin-1 abundance and promotes association of caveolin-1 and eNOS into inhibitory complexes, thereby decreasing NO production (65). Statins have been shown to reduce caveolin-1 abundance and decrease its inhibitory action on both basal and agonist-stimulated eNOS activity.

Another potential mechanism by which statins may improve endothelial function is through their antioxidant effects. For example, statins enhance endothelium-dependent relaxation by inhibiting the production of reactive oxygen species (ROS), such as superoxide and hydroxy radicals, from aortas of cholesterol-fed rabbits (66). Although lipid lowering by itself can lower vascular oxidative stress (67), some of these antioxidant effects of statins appear to be cholesterol-independent. For example, statins attenuate angiotensin II–induced free radical production in vascular smooth muscle cells (SMCs) by inhibiting Rac1-mediated NADH oxidase activity and downregulating angiotensin AT1 receptor expression (68) (Figure 4). Because NO is scavenged by ROS, these findings indicate that the antioxidant properties of statins may also contribute to their ability to improve endothelial function (49, 50).

## STATINS AND ENDOTHELIAL PROGENITOR CELLS

Statins have also been found to increase the number of circulating endothelial progenitor cells (EPCs) (69). EPCs augment ischemia-induced neovascularization (70), accelerate re-endothelialization after carotid balloon injury (71, 72) and improve postischemic cardiac function (73). Indeed, statins induce angiogenesis by promoting the proliferation, migration, and survival of circulating EPCs (74). In patients with stable coronary artery disease, administration of statins for four weeks augmented the number of circulating EPCs and enhanced functional capacity in patients with stable coronary artery disease (75). These findings agree with earlier data showing that statins rapidly mobilize EPCs from the bone marrow and accelerate vascular structure formation via activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase Akt and eNOS (55, 74, 76). These angiogenic effects were observed at lower concentrations of statins and were cholesterol-independent. At higher concentrations, statins appear to have an anti-angiogenic effect (77, 78), suggesting a biphasic effect of statins on angiogenesis (79). However, this suggestion remains controversial because higher doses of statins have also been shown to be angiogenic (80).
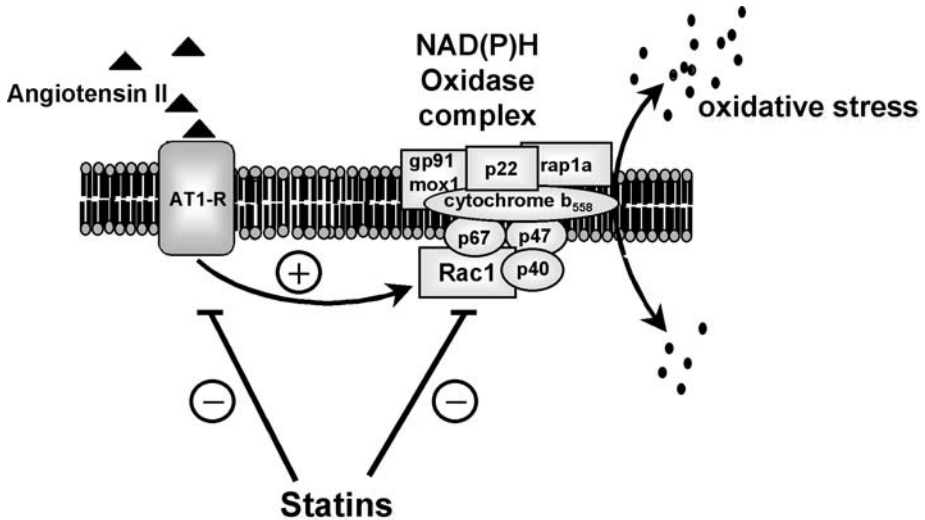
**Figure 4**    Antioxidative mechanisms of statins. The core NAD(P)H oxidase comprises five components: p40$^{phox}$ (PHOX for phagocyte oxidase), p47$^{phox}$, p67$^{phox}$, p22$^{phox}$, and gp91$^{phox}$. In the resting cell (*left*), three of these five components, p40$^{phox}$, p47$^{phox}$, and p67$^{phox}$, exist in the cytosol as a complex. The other two components, p22$^{phox}$ and gp91$^{phox}$, are located in the membranes. When it is stimulated by angiotensin, the cytosolic component becomes heavily phosphorylated and the entire cytosolic complex migrates to the membrane. Activation requires the participation not only of the core subunits but also of two low-molecular-weight guanine nucleotide-binding proteins, Rac and Rap. During activation, Rac binds GTP and migrates to the membrane along with the core cytosolic complex. Treatment with statin downregulates AT1-receptor expression and inhibits Rac1 GTPase, a necessary component of the NAD(P)H oxidase complex.

## Statins and Smooth Muscle Proliferation

The proliferation of vascular SMCs is a central event in the pathogenesis of vascular lesions, including post-angioplasty restenosis, transplant arteriosclerosis, and veinous graft occlusion (81). Recent studies have shown that statins attenuate vascular proliferative disease, such as transplant-associated arteriosclerosis (81). In contrast to atherosclerosis, transplant-associated arteriosclerosis is more dependent on immunological mechanisms as opposed to lipid disorders, although hypercholesterolemia exacerbates the immunologic process (82). Inhibition of isoprenoid but not cholesterol synthesis by statins decreased PDGF-induced DNA synthesis in vascular SMCs (39, 83). Treatment with statins decreased PDGF-induced Rb hyperphosphorylation and cyclin-dependent kinases (cdk)-2, -4, and -6 activities. This correlated with increases in the level of Cdk inhibitor, p27$^{Kip1}$, without concomitant changes in p16$^{INK4}$, p21$^{Waf1}$, or p53 levels. These findings

indicate that statins inhibit vascular SMC proliferation by arresting cell cycle between the G1/S phase transition. It remains to be determined whether the up-regulation of p27$^{Kip1}$ is responsible for the cell cycle arrest and whether there are differences between different statins in terms of p27$^{Kip1}$.

Because the small GTP-binding proteins, Ras and Rho, require posttransla-tional modification for membrane localization and activity and are implicated in cell cycle regulation, they are likely targets for the direct antiproliferative vas-cular effects of statins. Ras can promote cell cycle progression via activation of the MAP kinase pathway (84), whereas RhoA causes cellular proliferation through destabilizing p27$^{Kip1}$ protein (85). Interestingly, inhibition of vascular SMC proliferation by statins was reversed by GGPP, but not FPP or LDL-C (39). Indeed, direct inhibition of RhoA by *Clostridium botulinum* C3 transferase, which ADP-ribosylates and inactivates RhoA, or by a dominant-negative RhoA mu-tant increased p27$^{Kip1}$ and inhibited Rb hyperphosphorylation and SMC prolif-eration following PDGF stimulation. Taken together, these findings indicate that RhoA mediates PDGF-induced SMC proliferation and that inhibition of RhoA by statins is the predominant mechanism by which statins inhibit vascular SMC proliferation.

## STATINS AND PLATELET FUNCTION

Platelets play a critical role in the development of acute coronary syndromes (86). Circulating platelets are associated with mural thrombus formation at the site of plaque rupture and vascular injury (87, 88). Hypercholesterolemia is asso-ciated with increases in platelet reactivity (89). These abnormalities are linked to increases in the cholesterol/phospholipid ratio in platelets. Other potential mechanisms include increases in thromboxane A$_2$ (TXA$_2$) biosynthesis (90), platelet $\alpha_2$-adrenergic receptor density (91), and platelet cytosolic calcium (92).

Statins have been shown to influence platelet function, although the precise mechanisms involved are not fully understood (93, 94). One of the well-character-ized effects of endothelial NO is the inhibition of platelet aggregation (45). Statin-mediated upregulation of eNOS has been shown to be associated with downregulation of markers of platelet reactivity (95). Potential additional mech-anisms include a reduction in the production of TXA$_2$ and modifications in the cholesterol content of platelet membranes (96, 97). The cholesterol content of platelet and erythrocyte membranes is reduced in patients taking statin therapy. This may lead to a decrease in the thrombogenic potential of these cells. In-deed, animal studies suggest statin therapy inhibits platelet deposition on dam-aged vessels and reduces platelet thrombus formation (87, 98). Furthermore, in vitro experiments have demonstrated that statins inhibit tissue factor expression by macrophages, thereby potentially reducing thrombotic events in the vascular wall (99).

## STATINS AND PLAQUE STABILITY

Plaque rupture is a major cause of acute coronary syndromes (43, 100, 101). The atherosclerotic lesion contains highly thrombogenic materials in the lipid core that are separated from the bloodstream by a fibrous cap (102). Fissuring, erosion, and ulceration of the fibrous cap eventually lead to plaque rupture and ensuing thrombosis (101). Collagen is the main component of fibrous caps and is responsible for their tensile strength. Because macrophages are capable of degrading the collagen-containing fibrous cap, they play an important role in the development and subsequent stability of atherosclerotic plaques (103, 104). Indeed, degradation of the plaque matrix appears to be most active in macrophage-rich regions (43, 100). Secretion of proteolytic enzymes, such as metalloproteinases (MMPs), by activated macrophages may weaken the fibrous cap, particularly at the "vulnerable" shoulder region where the fibrous cap joins the arterial wall (105, 106). Weakened fibrous caps lead to plaque instability, rupture, and ensuing thrombosis, which ultimately present as acute coronary syndromes (43, 101, 107).

Lipid lowering by statins may contribute to plaque stability by reducing plaque size or by modifying the physiochemical properties of the lipid core (108, 109). However, changes in plaque size by lipid lowering tend to occur over extended time and are quite minimal as assessed by angiography. Rather, the clinical benefits from lipid lowering are probably due to decreases in macrophage accumulation in atherosclerotic lesions and inhibition of MMP production by activated macrophages (99). Indeed, statins inhibit the expression of MMPs and tissue factor by cholesterol-dependent and -independent mechanisms (99, 108, 110), with the cholesterol-independent or direct macrophage effects occurring at a much earlier time point. The plaque-stabilizing properties of statins, therefore, are mediated through a combined reduction in lipids, macrophages, and MMPs (111). These effects of statins may reduce the incidence of acute coronary syndromes by lessening the propensity for plaque to rupture and may explain the rapid time course of event reduction in patients at high risk for recurrent coronary ischemia in the MIRACL (112) and PROVE-IT trials (113).

## STATINS AND VASCULAR INFLAMMATION

Atherosclerosis is a complex inflammatory process that is characterized by the presence of monocytes or macrophages and T lymphocytes in the atheroma (114, 115). Inflammatory cytokines secreted by these macrophages and T lymphocytes can modify endothelial function, SMC proliferation, collagen degradation, and thrombosis (43). An early step in atherogenesis involves monocyte adhesion to the endothelium and penetration into the subendothelial space (115). Recent studies suggest that statins possess antiinflammatory properties owing to their ability to reduce the number of inflammatory cells in atherosclerotic plaques (96). The mechanisms have yet to be fully elucidated but may involve inhibition of adhesion

molecules, such as intercellular adhesion molecule-1 (ICAM-1), which are involved in the recruitment of inflammatory cells (116). Furthermore, statins attenuate P-selectin expression and leukocyte adhesion in normocholesterolemic animals by increasing endothelial NO production (117, 118). This cholesterol-independent effect of statins was absent in eNOS-deficient mice, suggesting that eNOS mediated the vascular protective effects of statins (119).

The activation of T-lymphocytes and the control of the immune response are mediated by the major histocompatibility complex class II (MHC-II) and CD40/CD40L. Under physiological conditions, antigen-presenting cells express MHC-II constitutively, whereas the induction of interferon gamma (INF-$\gamma$) leads to an increase of MHC-II expression in numerous cells, including human endothelial cells and monocytes. An important regulator in this pathway is the transactivator CIITA. Statins inhibit MHC-II expression on endothelial cells and monocyte-macrophages via inhibition of the promotor IV of the transactivator CIITA and thereby repress MHC-II-mediated T cell activation (120). In addition, statins have been shown to decrease CD40 expression and CD40-related activation of vascular cells (121).

A clinical marker of inflammation is high-sensitivity C-reactive protein (hs-CRP) (122). hs-CRP is an acute phase reactant that is produced by the liver in response to proinflammatory cytokines, such as interleukin-6 (IL-6), and reflects low-grade systemic inflammation (123). Elevated levels of hs-CRP have been shown to be predictive of increased risk for coronary artery disease (CAD) in apparently healthy men and women (124, 125). hs-CRP is elevated in patients with CAD, coronary ischemia and myocardial infarction compared with normal subjects (126). It has been suggested that CRP could also contribute to the development of atherosclerosis by binding to modified LDL-C within atherosclerotic plaques (127, 128). Once CRP becomes bound, it activates complement, which has been shown to play a role in promoting atherosclerotic lesion progression (129). Furthermore, CRP has been shown to induce plasminogen activator inhibitor (PAI)-1 expression and complement activation, increase the expression of cellular adhesion molecules, and decrease eNOS expression, leading to propensity for thrombosis, inflammation, and endothelial dysfunction. Indeed, transgenic overexpression of human CRP in transgenic mice leads to increased thrombosis and vascular inflammation following arterial injury (130). However, further studies are needed to fully elucidate the role CRP plays in atherosclerosis and cardiovascular risk.

Statin therapy lowers hs-CRP levels in hypercholesterolemic patients (122, 131, 132). In the CARE trial, statins significantly decreased plasma hs-CRP levels over a five-year period in patients who did not experience recurrent coronary events (133, 134). Similarly, an analysis of baseline and one-year follow-up from the AFCAPS/TexCAPS demonstrated that hs-CRP levels were reduced in statin-treated patients who were free of acute major coronary events (122). Furthermore, preliminary data from the Pravastatin Inflammation/CRP Evaluation (PRINCE) study confirm that statin therapy can significantly reduce serum hs-CRP levels in primary and secondary prevention populations (135). Following 24 weeks of therapy with a statin, the hs-CRP level was reduced by approximately 13% in primary and

secondary prevention populations, whereas placebo treatment of subjects in the primary prevention arm of the study had no effect. These studies, therefore, indicate that statins are effective in decreasing systemic and vascular inflammation. However, any potential clinical benefits conferred by the lowering hs-CRP are difficult to separate from that of the lipid-lowering effects of statins without performing further clinical studies. Perhaps the ongoing randomized placebo-controlled Jupiter Trial, which is enrolling patients with modest LDL-C (<130 mg/dl) and elevated hs-CRP (>2 mg/dl), will help address the question of whether CRP is an additional nonlipid-associated cardiovascular risk factor that can be modified by statin therapy.

## EFFECTS OF STATINS ON THE MYOCARDIUM

Cardiac hypertrophy is an adaptive response of the heart to pressure overload. In the myocardium, the small GTP-binding proteins, Ras, Rho, and Rac, and oxidative stress are involved in the hypertrophic response (136, 137). Indeed, recent animal studies suggest that a phagocyte-type NADPH oxidase may be a relevant source of ROS in the myocardium (138–140). NADPH oxidase-dependent ROS production appears to be involved in cardiac hypertrophy in response to pressure overload (140, 141), stretch (142), angiotensin II-infusion (139, 143), and $\alpha$-adrenergic stimulation (144). In the cardiomyocytes, three of its five components, $p40^{phox}$(PHOX for phagocyte oxidase), $p47^{phox}$, and $p67^{phox}$, exist in the cytosol, forming a complex (Figure 4). The other two components, $p22^{phox}$ and $gp91^{phox}$, are bound to the membranes. Various stimuli lead to the phosphorylation of the cytosolic components, and the entire cytosolic complex then migrates to the membrane. Importantly, not only the core subunits but also two low-molecular-weight guanine nucleotide-binding proteins, Rac1 and Rap, are required for activation. During activation, Rac1 binds GTP and migrates to the membrane with the core cytosolic complex. Therefore, Rac1 is critically involved in the activation of cardiovascular NADPH oxidase. Recent evidence both from animal and from human studies indicates that in failing myocardium, upregulation of Rac1 and $p47^{phox}$ membrane protein expression, as well as increased Rac1-GTPase activity, may resemble the underlying mechanisms for increased oxidase activity and may represent a novel therapeutic target for statin therapy.

Although the main impact of statin therapy in cardiovascular disease appears to be predominantly vascular, recent animal and human studies suggest that statins may also have direct beneficial effects on the myocardium. Because Rac1 is required for NADPH oxidase activity and cardiac hypertrophy is mediated, in part, by myocardial oxidative stress, it is likely that statins could inhibit cardiac hypertrophy through an antioxidant mechanism involving inhibition of Rac1 geranyl-geranylation. Indeed, statins inhibit angiotensin II–induced oxidative stress and cardiac hypertrophy in rodents (145). This has also been observed in clinical studies where statins inhibit cardiac hypertrophy in humans with hypercholesterolemia

(146). NADPH-oxidase-mediated ROS are increased in left ventricular myocardium from patients with heart failure and correlate with an increased activity of Rac1 GTPase, and oral statin treatment is able to decrease Rac1 function in the human heart (147).

The development of congestive heart failure (CHF), a common sequela of decompensated cardiac hypertrophy, is a major cause of death and morbidity in the Western world. Several lines of evidence suggest that statins may emerge as a novel treatment option for patients with CHF. Retrospective analysis of the large statin trials, such as the 4S, suggests that statins reduce the incidence and morbidity of heart failure (148). Second, patients with heart failure are characterized by increased vascular tone and endothelial dysfunction (149), which may be improved by statin therapy, irrespective of serum cholesterol levels. Third, statins have proven to preserve cardiac function in animal models of myocardial hypertrophy and heart failure, such as aortic banding, myocardial infarction, and several transgenic models (145, 150–152).

In a recent prospective, double blind, placebo-controlled study, patients with symptomatic, nonischemic, dilated cardiomyopathy were randomly divided into two groups receiving statin or placebo for 14 weeks (153). Although patients receiving statins exhibited a modest reduction in serum cholesterol level compared to patients receiving placebo, these patients demonstrated a significant improvement in exercise endurance, as exhibited by a lower New York Heart Association functional class compared to patients receiving placebo. This corresponded to improved left ventricular ejection fraction in the statin group ($33 \pm 4$ to $41 \pm 4\%$, $P < 0.01$), but not in the placebo group. The improvements in their exercise endurance and heart function were in addition to the improvements already observed with two current treatments for heart failure, beta-blockers and ACE inhibitors. Furthermore, plasma concentrations of tumor necrosis factor alpha (TNF-$\alpha$), IL-6, and brain natriuretic peptide (BNP) were lower in the statin group compared to the placebo group. This study indicates that short-term statin therapy improves cardiac function, neurohormonal imbalance, and symptoms associated with idiopathic dilated cardiomyopathy. These observations were confirmed in a second study using cerivastatin (154). These findings suggest that statins may have therapeutic benefits in patients with heart failure irrespective of serum cholesterol levels or atherosclerotic heart disease.

## STATINS AND ISCHEMIC STROKE

Although myocardial infarction is closely associated with serum cholesterol levels, neither the Framingham Heart Study nor the Multiple Risk Factor Intervention Trial (MRFIT) demonstrated significant correlation between ischemic stroke and serum cholesterol levels (12, 13). An intriguing result of large clinical trials with statins is the reduction in ischemic stroke (155). For example, the recent Heart Protection Study (HPS) shows a 28% reduction in ischemic strokes in over

20,000 people with cerebrovascular disease or other high-risk conditions (156). The proportional reductions in stroke were approximately one quarter in all subcategories studied, including those aged over 70 years at entry and those presenting with different levels of blood pressure or lipids, even when the pretreatment LDL-C was below 3.0 mmol/L (116 mg/dl). Thus, the findings of these large statin trials raise the interesting question of how a class of cholesterol-lowering agents can reduce ischemic stroke when ischemic stroke is not related to cholesterol levels. It appears likely that there are cholesterol-independent effects of statins, which are beneficial for ischemic stroke. Some of these beneficial effects may relate to the effects of statins on endothelial and platelet function.

Cerebrovascular tone and blood flow are regulated by endothelium-derived NO (157). Mutant mice lacking eNOS (eNOS$^{-/-}$) are relatively hypertensive and develop greater proliferative and inflammatory response to vascular injury (158). Indeed, eNOS$^{-/-}$ mice develop larger cerebral infarcts following cerebrovascular occlusion (159). Thus, the beneficial effects of statins in ischemic stroke may, in part, be due to their ability to upregulate eNOS expression and activity (32, 55). For example, mice that were prophylactically treated with statins for up to two weeks, have 25%–30% higher cerebral blood flow and 50% smaller cerebral infarct sizes following cerebrovascular occlusion (160). No increase in cerebral blood flow or neuroprotection was observed in eNOS$^{-/-}$ mice treated with statins, indicating that the upregulation of eNOS accounts for most, if not all, of the neuroprotective effects of these agents. Interestingly, treatment with statins did not affect blood pressure or heart rate before, during, or after cerebrovascular ischemia and did not alter serum cholesterol levels in mice, consistent with the cholesterol-independent, neuroprotective effects of statins.

In addition to increases in cerebral blood flow, other beneficial effects of statins are likely to occur that can impact on the severity of ischemic stroke. For example, statins attenuate P-selectin expression and leukocyte adhesion via increases in NO production in a model of cardiac ischemia and reperfusion (161, 162). Others have reported that statins upregulate tissue-type plasminogen activator (t-PA) and downregulate plasminogen activator inhibitor (PAI)-1 expression through a similar mechanism involving inhibition of Rho geranylgeranylation (57). Thus, the absence of neuroprotection in eNOS-deficient mice emphasizes the importance of endothelium-derived NO in not only augmenting cerebral blood flow but also, potentially, in limiting the impact of platelet and white blood cell accumulation on tissue viability following ischemia. In humans, atherosclerosis of precerebral arteries causes stroke through plaque disruption and artery-to-artery thromboembolism, and, in contrast to the mouse models, statins exert additional stroke-protective effects in humans through their anti-atherosclerotic and plaque-stabilizing effects. Furthermore, the antiinflammatory actions and mobilization of endothelial progenitor cells of statins may also contribute to neuroprotection. It is therefore possible that statins have contributed to the decrease in the incidence of ischemic strokes in clinical trials, in part, by reducing cerebral infarct size to levels that were clinically unappreciated.

## STATINS AND DEMENTIA

Recent epidemiological reports suggest that statins might be protective for Alzheimer's disease, and for other types of dementia (163). Dementia is a syndrome of chronic or progressive nature with multiple disturbances of higher cortical functions. This syndrome occurs in Alzheimer's disease, in cerebrovascular disease (i.e., multi-infarct dementia), and in other conditions primarily or secondarily affecting the brain. Alzheimer's disease is related to the effects of $\beta$-amyloid, a peptide that accumulates in the brain, causing neurotoxicity and neurodegeneration. Experimental and clinical studies suggest that there is a pathophysiologic relation between $\beta$-amyloid and cholesterol levels. Elevated $\beta$-amyloid levels and the $\varepsilon4$ allele of the apolipoprotein E (APOE4) are risk factors for Alzheimer's disease (164). In addition, APOE4 is correlated with increased risk for atherosclerosis and amyloid plaque formation (165, 166). Observational studies revealed that an elevated serum cholesterol level is a risk factor for Alzheimer's disease (167). Statins, regardless of their brain availability, have been suggested to induce alterations in cellular cholesterol distribution in the brain. Such cholesterol-independent effects of statins might be mediated via NO or ApoE (168, 169). A cross-sectional analysis of three hospital databases by Wolozin and colleagues suggested that the prevalence of Alzheimer's disease in patients taking statins is 60% lower in comparison to patients taking other medications used in the treatment of cardiovascular diseases (170). A nested case control study based on the UK-based General Practice Research Database showed that among individuals 50 years and older with a statin therapy, the risk for developing dementia was significantly reduced, independent of their lipid status (171). Furthermore, other lipid-lowering agents had no influence on the risk of developing dementia in this population. The systemic vascular protective effects of statin treatment are very likely to contribute to their beneficial effects, especially on vascular forms of the dementia syndrome. However, the precise underlying molecular mechanisms are poorly understood. Indeed, the results of the recent HPS and Prospective Study of Pravastatin in the Elderly at Risk (PROSPER) trials do not demonstrate the efficacy of statins in slowing cognitive decline and dementia (9, 172).

Recent evidence has stimulated the discussion of statins as potential novel antiinflammatory and vascular protective agents for the treatment of other cerebral diseases, such as multiple sclerosis and depression. Oral atorvastatin was shown to prevent chronic and relapsing experimental autoimmune encephalomyelitis, a CD4(+) Th1-mediated central nervous system (CNS) demyelinating disease model of multiple sclerosis (173). Statin induced STAT6 phosphorylation and secretion of Th2 cytokines and transforming growth factor (TGF)-$\beta$. Conversely, STAT4 phosphorylation was inhibited and secretion of Th1 cytokines was suppressed. Statin promoted the differentiation of Th0 cells into Th2 cells. In adoptive transfer, these Th2 cells protected recipient mice from induction of autoimmune encephalomyelitis. Statin reduced CNS infiltration and mMHC-II expression. Treatment of microglia inhibited IFN-$\gamma$-inducible transcription at multiple

MHC-II transactivator (CIITA) promoters and suppressed MHC-II upregulation. Statin suppressed IFN-$\gamma$-inducible expression of CD40, CD80, and CD86 co-stimulatory molecules as well as antigen-specific T cell activation. Similarly, a second study shows that oral statin inhibited the development of actively induced chronic CD4^{+}T cell–mediated experimental autoimmune encephalomyelitis in a preventive and therapeutic fashion and significantly reduced the inflammatory infiltration into the CNS (174). This potentially therapeutic effect was associated with downregulation of Th1 immune response. In addition, similar to the effects of statins in vascular SMCs, statins inhibited the cell cycle of human antigen–specific T cells. Thus, statins exert pleiotropic immunomodulatory effects involving both antigen presenting cells (APC) and T cell compartments, and they may be beneficial for multiple sclerosis and other Th1-mediated autoimmune diseases.

A possible association between lipid-lowering drug therapy and psychological well-being has been an issue of debate. A recent nested case-control analysis of the United Kingdom General Practice Research Database revealed that the use of statins and other lipid-lowering drugs is not associated with an increased risk of depression or suicide (175). On the contrary, individuals with current statin use may have a lower risk of developing depression, an effect that could be explained by improved quality of life owing to decreased risk of cardiovascular events or more health consciousness in patients receiving long-term treatment. Furthermore, another comparison of patients who had continuous use of statins with the patients who did not use any cholesterol-lowering drugs showed that statin use was associated with lower risk of abnormal depression scores, anxiety, and hostility, after adjustment for the propensity for statin use and potential confounders. Interestingly, the beneficial psychological effects of the statins appeared to be independent of the drugs' cholesterol-lowering effects (176).

## CLINICAL TRIALS WITH STATINS: EVIDENCE FOR PLEIOTROPY

Because serum cholesterol level is strongly associated with coronary heart disease, it has been generally assumed that cholesterol reduction by statins is the predominant, if not the only mechanism, underlying their beneficial effects. Data from a meta-analysis of lipid-lowering trials suggest lipid modification alone accounts for the clinical benefits associated with statin therapy. Indeed, the slope of the relationship between cholesterol reduction and mortality risk reduction was the same for statins and nonstatins, whereas the mortality risk reductions realized over statin treatment periods of two years and longer were found to be a consequence of cholesterol reduction alone (Figure 5, left panel). However, this type of meta-analysis does not take into account the differences in terms of the length of the individual trials with respect to cardiovascular benefits. Some of the nonstatin lipid-lowering trials, such as the Lipid Research Clinic–Coronary Primary Prevention Trial (LRC-CPPT) using the bile acid resin cholestyramine (177), or the
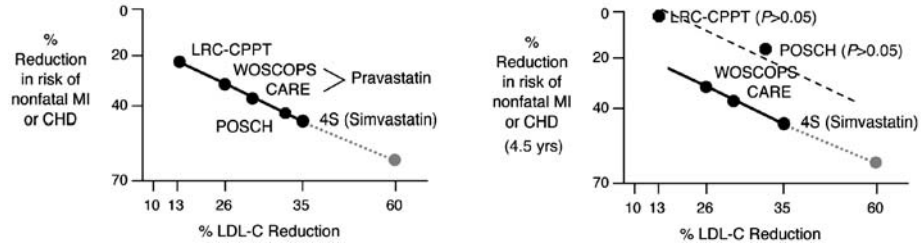
**Figure 5**  Relationship between LDL-C reduction and risk of cardiovascular events. (*Left panel*) Decrease in LDL-C (% reduction) is correlated with reduction in risk of nonfatal myocardial infarctions (MI) or coronary heart disease (CHD) among statin (WOSCOPS, CARE, and 4S) and nonstatin (LRC-CPPT and POSCH) trials. Note that the relationship (*slope*) holds between statin and nonstatin trials, suggesting that the beneficial effects of statins are likely due to only cholesterol lowering. (*Right panel*) Decrease in LDL-C (% reduction) is correlated with reduction in risk of nonfatal myocardial infarctions (MI) or coronary heart disease (CHD) among statin (WOSCOPS, CARE, and 4S) and nonstatin (LRC-CPPT and POSCH) trials after 4.5 years of treatment. Note that the nonstatin trials (LRC-CPPT and POSCH; *dashed lines*) show less cardiovascular benefits than statin trials (WOSCOPS, CARE, and 4S) and they no longer fall on the same slope (*solid line*).

Program on the Surgical Control of the Hyperlipidemias (POSCH) using partial ileal bypass surgery (178), reported benefits after 7.4 and 9.7 years, respectively, whereas most of the statin trials showed benefits at much earlier time points (e.g., within 5 years). Thus, if one compares the benefits after five years for all lipid-lowering trials, one finds that the nonstatin trials no longer fall on the same slope of cholesterol:mortality risk reduction as do all of the statin trials (Figure 5, right panel). In fact, the benefits of cholesterol lowering after ileal bypass surgery in the POSCH study were not realized at 4.5 years, despite significant LDL-C reduction of 34% within the first 3 months after the surgical procedure. These results suggest that the beneficial effects of statins occur more rapidly and may not be entirely dependent on cholesterol reduction.

Despite the rapidity of benefits of statin therapy compared to other nonstatin lipid-lowering therapies, it is still difficult to prove that pleiotropic effects of statins are real. First, patients receiving statin therapy invariably will have reduced lipid levels and it is often difficult to separate the lipid- from the nonlipid-lowering effects of statins in clinical trials. Second, many effects of statins, such as improvement in endothelial function, decreased inflammation, increased plaque stability, and reduced thrombogenic response, could all be accounted for, to some extent, by lipid lowering. Third, the concentrations used to demonstrate the biological effects of statins in cell culture and animal experiments, especially with regards to inhibition of Rho geranylgeranylation but not PI3-kinase/Akt activation, appear to be much higher than what is prescribed clinically. Finally, both hydrophilic and lipophilic statins, which inhibit hepatic HMG-CoA reductase, appear

to exert similar cholesterol-independent effects, despite the relative impermeability of hydrophilic statins in vascular tissues. Thus, it appears that statins are very potent cholesterol-lowering agents and that reduction in cholesterol levels by statins contributes to many of their clinical benefits.

However, in the recent HPS and ASCOT trials, the relative risk reduction conferred by statin treatment was independent of the pretreatment lipid levels (9, 10). These large prospective trials raise the question of whether individuals with CHD could benefit from statin drugs independently of cholesterol levels. Interestingly, subgroup analyses of previous clinical trials suggested that the beneficial effects of statins could extend to mechanisms beyond cholesterol reduction. For example, subgroup analysis of the WOSCOPS and CARE studies indicate that despite comparable serum cholesterol levels among the statin-treated and placebo groups, statin-treated individuals have significantly lower risks for coronary heart disease compared to age-matched placebo-controlled individuals (5, 7). Indeed, when the statin treatment group was divided into quintiles of percentage LDL-C reduction, it was found that there was no difference in the 4.4-year coronary event rate for quintiles 2 through to 5 (LDL-C reductions of 23%–41%). Hence, there was no apparent association between coronary event rate and the level of LDL-C reduction. Furthermore, meta-analyses of cholesterol-lowering trials suggest that the risk of myocardial infarctions in individuals treated with statins is significantly lower compared to individuals treated with other cholesterol-lowering agents or modalities despite comparable reduction in serum cholesterol levels in both groups (179). For example, application of the Framingham risk score to WOSCOPS produced a coincidence between predicted and observed risk in the placebo group but underestimated the benefit of the pravastatin group by 31% (180).

**TABLE 1**   Statin pleiotropy

| Effect | Benefit |
| --- | --- |
| Increased synthesis of nitric oxide | Improvement of endothelial dysfunction |
| Inhibition of free radical release | |
| Decreased synthesis of endothelin-1 | |
| Inhibition of LDL-C oxidation | |
| Upregulation of endothelial progenitor cells | |
| Reduced number and activity of inflammatory cells | Reduced inflammatory response |
| Reduced levels of C-reactive protein | |
| Reduced macrophage cholesterol accumulation | Stabilization of atherosclerotic plaques |
| Reduced production of metalloproteinases | |
| Inhibition of platelet adhesion/aggregation | Reduced thrombogenic response |
| Reduced fibrinogen concentration | |
| Reduced blood viscosity | |

Finally, the lipophilic statins would be expected to penetrate cell membranes more effectively than the more hydrophilic statins, causing more side effects but at the same time eliciting more pleiotropic effects. However, the observation that hydrophilic statins have similar pleiotropic effects as lipophilic statins puts into question whether there are really any cholesterol-independent effects of statins. Indeed, recent evidence suggests that some of the cholesterol-independent effects of these agents may be mediated by inhibition of hepatic HMG-CoA reductase leading to subsequent reduction in circulating isoprenoid levels (28). This hypothesis may help explain why hydrophilic statins, such as pravastatin and rosuvastatin, are still able to exert cholesterol-independent benefits on the vascular wall without directly entering vascular wall cells. In this respect, the word pleiotropic probably does not reflect the hepatic versus nonhepatic effects of these agents.

## SUMMARY

Statins exert many pleiotropic effects in addition to the lowering of serum cholesterol levels. These additional properties include beneficial effects on endothelial function and blood flow, decreasing LDL-C oxidation, enhancing the stability of atherosclerotic plaques, inhibiting vascular smooth muscle proliferation and platelet aggregation, and reducing vascular inflammation (Table 1). Recent evidence suggest that most of these effects are mediated by statin's inhibitory effect on isoprenoid synthesis. In particular, inhibition of Rho GTPases in vascular wall cells by statins leads to increased expression of atheroprotective genes and inhibition of vascular SMC proliferation. It remains to be determined which of and to what extent these pleiotropic effects account for the clinical benefits of statin therapy beyond cholesterol lowering.

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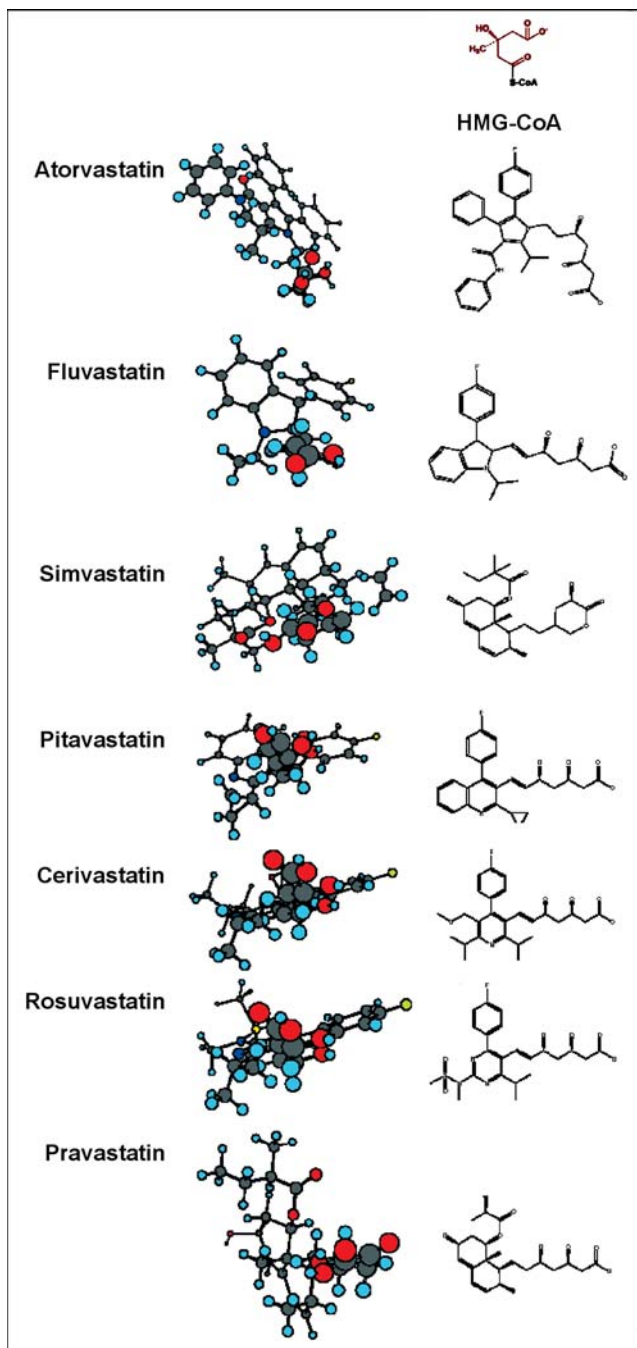
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**Figure 1** Structural basis of HMG-CoA reductase inhibition by statins. The active forms of statins resemble the cholesterol precursor, HMG-CoA (*right panels*). All statins share the HMG-like moiety and competitively inhibit the reductase by the similar mechanism but have distinct pharmacologic and pharmacodynamic properties related to their chemical structures (*left panels*, *ball and stick* graphs: *black*, carbon; *red*, oxygen; *light blue*, hydrogen; *dark blue*, nitrogen).

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