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Forum Review Article

Statins in Endothelial Signaling and Activation

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Abstract

The beneficial effects of statins, the most widely prescribed class of drugs in the world, are now recognized to extend well beyond their lipid-lowering properties. Through a combination of both distinct and interdependent effects on endothelial cell (EC) Rho GTPase regulation, NAPDH oxidase activity, NO bioavailability, and differential gene expression, statins confer significant protection of the vasculature. Abundant *in vitro* data, in addition to myriad reports relying on a range of animal models, now firmly support the idea that these drugs may serve as novel and effective therapeutic agents in a variety of disease states characterized by vascular dysfunction. *Antioxid. Redox Signal.* 11, 811–821.

Introduction

The statins are a class of 3-hydroxy-3-methylglutaryl-coenzymeA (HMG-CoA) reductase inhibitors used clinically for their ability to reduce serum cholesterol levels as well as for their beneficial effects on the morbidity and mortality due to coronary artery disease. However, these drugs are now widely recognized to have pleiotropic properties including direct effects on endothelial function through the inhibition of reactive oxygen species (ROS) generation and the upregulation of endothelial nitric oxide synthase (eNOS). The idea that statins may have clinical effects independent of cholesterol-synthesis inhibition was initially suggested by the results of the first large clinical trials involving these drugs. Although the landmark Scandinavian Simvastatin Survival Study (4S) and West of Scotland Coronary Prevention Study (WOSCOPS) (1, 72) both identified a significant mortality benefit in patients with hypercholesterolemia, a close examination of these data revealed that the mortality benefits of statin treatment were evident early, before the anticipated time course of atherosclerotic plaque regression due to lowered serum cholesterol levels (2). Subsequently, the ability of statins to improve vascular function before effecting measurable changes in serum cholesterol in healthy volunteers has been reported (54), as well as evidence of increased morbidity and mortality in patients hospitalized with an acute myocardial infarct who did not receive a statin within the first 24 h of admission (30). More recently, newer generations of cholesterol- reducing medications such as torcetrapib, a cholesterol ester transfer protein inhibitor, failed to demonstrate the same clinical benefits as statins, despite their ability to reduce serum cholesterol levels significantly, further implicating a cholesterol-independent mechanism of statin effects (9, 13). As our appreciation of these effects continues to grow, their potential clinical implications have generated intense interest. This review will address our current understanding of the direct of effects of statins on endothelial cell (EC) signaling and function and their potential clinical relevance.

Statins and Rho GTPase Regulation

The statins inhibit HMG-CoA reductase, thereby preventing the prenylation of HMG-CoA, a posttranslational modification that culminates in either farnesylation, the addition of a 15-carbon side chain, or geranylgeranylation, the addition of two 20-carbon side chains. One of the downstream products of farnesylation is cholesterol (38); however, a number of other proteins, including the Rho family of small GTPases, are dependent on geranylgeranylation for membrane translocation and subsequent activation (89). While the mechanisms of statin effects on the endothelium are undeniably complex, the inhibition of the small GTPases, RhoA and Rac1, is clearly a key factor, although even this effect of statins is not so straightforward. In particular, we previously reported evidence of differential effects of statins on EC Rho GTPase activation dependent on the cellular localization of these molecules (43).

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The Rho GTPases are known to cycle within cells between what are often referred to as an active, or GTP-bound state, and an inactive, or GDP-bound state. This cycling occurs in part through the effects of specific mediators, including a variety of GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) (22). Although it is the case that geranylgeranylation is requisite for Rho GTPase attachment to the cell membrane, which in turn promotes GTP-binding at these sites, we and others confirmed increased GTP-bound RhoA and Rac1 intracellularly in statin-treated ECs despite the inhibition of membrane translocation (15, 18, 43, 84). Nonetheless, the inhibition of Rho GTPase membrane localization is frequently used as a surrogate for Rho GTPase inactivation. Although a number of authors have postulated that GTP-bound Rho GTPases that are not prenylated (and therefore not membrane bound) are effectively nonfunctional, some evidence to the contrary exists, including signaling *via* p38, a mitogen-activating protein kinase (MAPK), by Rac-GTP, despite the inhibition of prenylation by risendronate, a bisphosphonate (18). Nonetheless, the significance of increased GTP loading of cytosolic Rho GTPases by statins is essentially unknown. One thing, however, is clear: the nomenclature used to designate "activation" of Rho GTPases must be clarified and standardized.

The mechanisms underlying increased GTP loading of Rho GTPases by statins, despite the inhibition of geranygeranylaton, are unclear. Evidence suggests that this may be the result of decreased association with specific negative regulators, such as Rho guanine dissociation inhibitor (GDI) in the case of Rac (15), which could be accounted for by the fact that the geranygeranylation of Rac is required for its association with Rho GDI (17). However, this can at best represent only part of the story, as geranygeranylation in general favors Rac-GTP loading.

Aside from their paradoxic effects on GTP loading of Rho GTPases, notable time-dependent effects are found of statins on EC Rho GTPase regulation. In particular, inhibition of geranylgeranylation with subsequent translocation of Rho GTPases from the membrane to the cytosol has been shown to occur within 1 h of statin treatment (23), and we have observed evidence of RhoA inhibition characterized by dynamic actin cytoskeletal rearrangement within 2 h of simvastatin treatment of ECs (14). Despite these early effects of statins on prenylation, we found evidence of increased GTP loading of Rho GTPases in ECs only after prolonged (16 h) treatment with simvastatin (43).

Statins and Cytoskeletal Rearrangement

As suggested above, one significant consequence of the inhibition of Rho GTPase geranylgeranylation by statins is dynamic rearrangement of the EC actin cytoskeleton (43). These changes occur in a time-dependent manner and are characterized both by an attenuation of transcellular actin stress fibers and by an augmentation of actin distributed around the cell periphery (Fig. 1). Although the diminution of transcellular stress fibers corresponds to early RhoA inhibition at the cell membrane, the role of Rac in statin-mediated EC cytoskeletal changes is unclear. In particular, in response to a number of other agonists, including sphingosine 1-phosphate (S1P), ATP, hepatocyte growth factor (HGF), and activated protein C (APC), we previously reported similar

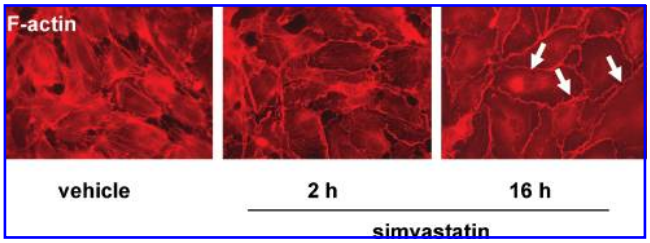

FIG. 1. Endothelial cytoskeletal rearrangement by statins. Immunofluorescent images of ECs treated with simvastatin (5 μM) demonstrate evidence of actin rearrangement within 2 h characterized by decreased transcellular stress fibers and enhanced cortical actin (arrows) compared with vehicle control cells. These changes are associated with decreased paracellular gaps and are even more pronounced at 16 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

changes in peripheral actin augmentation associated with increased GTP loading of Rac1 occurring rapidly in these settings (28, 35, 45, 60). However, the delayed time course of these effects on Rac1 by statins does not correspond to the early evidence of these particular EC cytoskeletal changes. This is notwithstanding the fact that, as previously noted, it is unclear whether increased cytosolic Rac-GTP has any functional significance in the first place.

The characteristic EC cytoskeletal changes induced by statins are associated with decreased paracellular gaps and improved monolayer integrity. Functionally, these changes correspond to EC-barrier protection as measured both by transendothelial electrical resistance (TER) and by FITC-dextran flux across EC monolayers in response to barrier-disruptive agonists (14, 43). Moreover, we have found that simvastatin-mediated EC barrier protection can be partially replicated by Rho inhibition by using either a pharmacologic inhibitor of Rho kinase (Y27632) or by silencing RNA specific for RhoA (14). To a lesser extent, we also were able to demonstrate evidence of EC barrier protection related to Rac inhibition by using siRNA specific for Rac1.

Although these data support the idea that the inhibition of Rho GTPase geranylgeranylation with subsequent effects on cytoskeletal rearrangement is a critical determinant of statin-mediated EC barrier protection, an accurate interpretation of these findings must also take into account the role of Rho GTPases on EC signaling, independent of augmentation of the actin cytoskeleton (discussed below), as well as the fact that efforts to replicate statin effects through inhibition of Rho GTPases may ultimately be misguided, depending on the significance of statin-induced increases in cytosolic GTP loading of these proteins.

Notably, no evidence is apparent of increased TER at various time points of statin treatment of otherwise unstimulated EC monolayers. However, in response to specific barrier-disruptive agonists, including thrombin and lipopolysaccharide (LPS), statin pretreatment effects a significant attenuation of barrier disruption, as measured by both TER and FITC-dextran flux (Fig. 2). For this reason, we have characterized statin-mediated effects as consistent with EC barrier protection rather than with barrier enhancement. This is in contrast to other agonists we have studied, including S1P, ATP, and HGF, all of which induce enhanced barrier function under

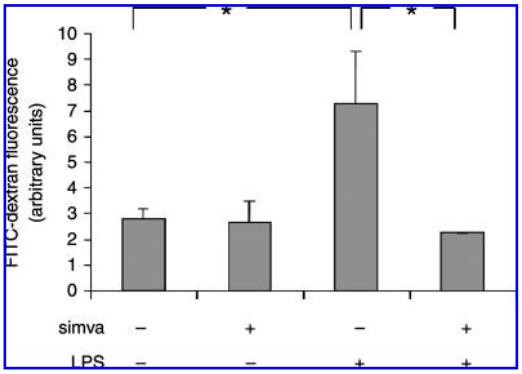

FIG. 2. Endothelial barrier protection by statins. EC permeability as measured by FITC-dextran flux across cells grown to confluence in transwell inserts and then subjected to LPS (100 ng/ml, 6 h) confirm significant protection conferred by simvastatin pretreatment (5 μM, 16 h before LPS) compared with LPS-treated controls ($^*p < 0.05$).

basal conditions implicating important differences with respect to their mechanisms of actions compared with statins.

Aside from actin cytoskeletal rearrangement, the effects of statins on EC Rho GTPase activity are also associated with several downstream consequences, including eNOS upregulation, NADPH oxidase inhibition, and differential gene expression. Collectively, these effects directly influence vascular function and contribute significantly to the vascular-protective properties of statins which have now been described in a variety of clinical settings.

Statins and NADPH Oxidase

The attenuation of superoxide generation by NADPH oxidase inhibition represents a distinct vascular-protective mechanism of statins. The EC NADPH oxidase complex consists of multiple components, including membrane-bound p22*phox* and Nox2, as well as cytosolic regulatory subunits p47*phox*, p67*phox*, and Rac1 (46, 57). Superoxide generation is induced on assembly of the NADPH complex, which then rapidly reacts with NO to produce peroxynitrite ($ONOO^-$) and promotes vascular dysfunction both directly and through consequent decreased NO bioavailability. Notably, a separate Nox isoform, Nox4, is also present in ECs but does not associate with known regulatory subunits, and evidence suggests that Nox4–based oxidase is constitutively active (4, 7, 61). The attenuation of EC Nox2-based NADPH oxidase activity by statins would be predicted by the inhibition of Rac1 geranygeranylation, which prevents membrane localization and subsequent complex assembly (Fig. 3). We confirmed this effect of statins both through the ability of simvastatin to attenuate LPS-induced EC superoxide generation and by evidence of Rac1 and p47*phox* translocation from the EC membrane to the cytosol in response to simvastatin treatment (14). Moreover, the attenuation of LPS-induced EC superoxide generation by statins can be replicated by using silencing RNA specific for Rac1. These effects of simvastatin are reversed by gerany geranyl-pyrophosphate (GGPP), indicating that NADPH oxidase inhibition is the result of inhibition of HMG-CoA reductase and gerany geranylation rather than the consequence of a nonspecific effect of statins.

NADPH oxidase is also inhibited by statins through the downregulation of the G protein–coupled angiotensin II type-1 (AT-1) receptor gene, resulting in decreased expression in ECs (85), as well as in both vascular smooth muscle cells and platelets (65, 87). Activation of this receptor by angiotensin II induces both protein kinase C (PKC) and Rac1 activation (71) and is an important mechanism of superoxide generation by

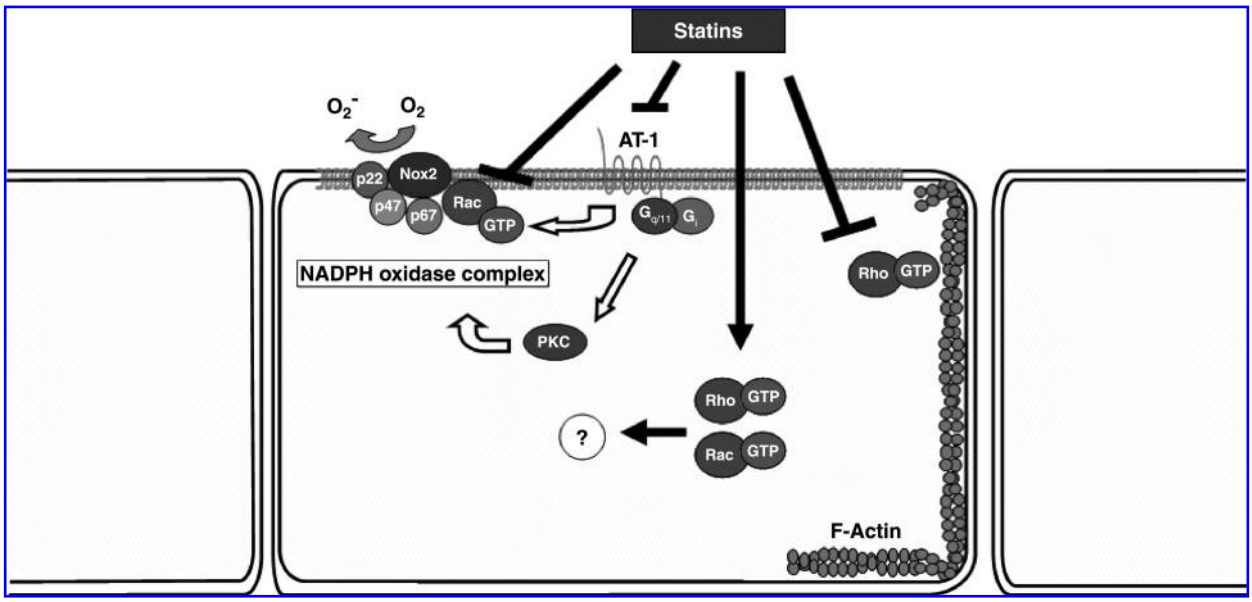

FIG. 3. Rho GTPase regulation and NADPH oxidase inhibition by statins. The inhibition of geranylgeranylation by statins prevents membrane translocation of Rho GTPases including Rho and Rac. Inhibition of RhoA geranylgeranylation results in dynamic rearrangement of the EC actin cytoskeleton. Inhibition of Rac1 geranylgeranylation prevents assembly of the NADPH oxidase complex at the cell membrane, resulting in decreased ROS generation. Statins also downregulate the angiotensin II AT-1 receptor, which independently results in decreased NADPH oxidase activity. Of note, statins induce increased GTP-loading of cytosolic Rho GTPases, although the functional significance of this effect is unknown.

NADPH oxidase (86). Statin treatment results in a decreased half-life of AT-1 receptor mRNA, although it does not affect gene transcription levels (87). This effect has been shown to be independent of cholesterol levels and can be replicated by specific inhibition of geranylgeranyl-transferase. The endothelium-specific effects of statins on AT-1 signaling are suggested by the Endothelial Protection, AT1 Blockade and Cholesterol-Dependent Oxidative Stress (EPAS) trial (63). In this study, the effects of statins on endothelial function and gene expression in patients with coronary artery disease were found to be consistent with that induced by AT-1 receptor blockade through irbesartan. Both interventions were associated with improved endothelial function and significant effects on an endothelial expression quotient, calculated based on the mRNA expression of specific genes, including eNOS and NADPH oxidase, although the expression of these genes individually was not differentially expressed in the different study groups.

A direct link also may exist between cytoskeletal rearrangement induced by statins and their inhibition of NADPH oxidase. We previously reported decreased hyperoxia-mediated ROS production in ECs associated with the stabilization of actin filaments by phalloidin, whereas the use of actin disrupters, including cytochalasin D or latrunculin A, results in increased ROS production induced by hyperoxia (80). Moreover, we identified the actin-binding protein cortactin as an essential regulator of hyperoxia-induced EC ROS production through interactions with the regulatory component of the NADPH oxidase complex $p47^{phox}$, which is known to associate with actin in a variety of cell types (76, 78, 88). We also reported prominent translocation of cortactin to cell periphery that colocalizes with polymerized actin in ECs treated with statins (43). These data support the notion that direct effects on the actin cytoskeleton and specific cytoskeletal proteins by statins may themselves effect NADPH oxidase activity.

Finally, inhibition of NADPH oxidase by statins is independently associated with the barrier regulatory properties of these drugs. This is supported indirectly by evidence of barrier-disruption induced by superoxide as well as by direct evidence of both the inhibition of superoxide generation and EC barrier protection by silencing of Rac1, consistent with the inhibition of gerany geranylation by statins (14).

Statins and eNOS

NO synthesis is mediated by the various isoforms of NOS, including eNOS, an enzyme constitutively expressed in EC, as well as other cell types, chiefly responsible for vascular NO synthesis. Basal eNOS expression and activity may be affected by a variety of stimuli including differentially by oxidized low-density lipoproteins under various conditions (69) and enhancement by shear stress (16, 90) or in the lung under conditions of chronic hypoxia (55). In addition, although NO generation by eNOS may occur through a Ca^{2+}/calmodulin-dependent pathway, a distinct mechanism independent of intracellular Ca^{2+} levels and relevant to statin-mediated effects has also been described involving signaling by activation of the serine/threonine protein kinase Akt, also known as protein kinase B (16). NO affects vasodilatation through soluble guanylyl cyclase activation and increased cGMP (31, 42). Consistent with these effects, decreased NO activity is a determinant of endothelial dysfunction in a variety of clinical conditions including atherosclerosis. Accordingly, interventions that result in an increase in NO bioavailability would potentially be associated with clinical benefits in these settings.

Any one of a number of variables can affect alterations in NO bioavailability. Expression levels of eNOS and its phosphorylation independently determine NO synthesis (16, 29). Separately, ROS is a determinant of NO activity through its direct interaction with this molecule, as superoxide reacts with NO to produce peroxynitrite, and an attenuation of endothelium- dependent vasodilation can be affected in part by an inactivation of endothelium-derived NO by superoxide (66). Further physiologic evidence of this is provided by the findings of more pronounced endothelium-dependent relaxation in response to acetylcholine in aortas from $Nox2^{-/-}$ mice compared with aortas from control animals (39).

Increased eNOS activity induced by statins has been implicated as one mechanism of protection in a number of disparate animal models including rat models of myocardial ischemia (56) and cirrhotic portal hypertension (79), as well as diabetic microangiopathic hindlimb ischemia (32), and ischemic stroke (20) in mice. In this last model, the importance of increased eNOS activity in statin-mediated protection was strongly supported by evidence of a lack of statin protection in eNOS-deficient mice. Separately, in the aortas of mice treated with atorvastatin for 14 days, eNOS activity and mRNA expression were found to be increased more than twofold (53), and in human ECs, statins have been shown both to upregulate eNOS directly (52) and to attenuate the downregulation of eNOS induced by either oxidized low-density lipoprotein (51) or thrombin stimulation (24).

As suggested by these reports, the mechanisms underlying increased eNOS bioavailability by statins are twofold, effecting both increased expression and increased activity, and rely on both cholesterol-dependent and cholesterol-independent processes (Fig. 4). Increased eNOS mRNA expression is the result of augmented mRNA stability rather than increased gene transcription and is both reversed by the coadministration of GGPP, implicating the role of gerany geranylation inhibition, and independent of extracellular cholesterol levels (51). Moreover, increased eNOS expression by statins has been linked to the inhibition of RhoA geranylgeranylation as Rho inhibition by *Clostridium botulinum* C3 transferase or overexpression of a dominant-negative N19RhoA mutant replicates these effects, whereas Rho activation induced by *Escherichia coli* cytotoxic necrotizing factor-1 is also associated with decreased eNOS expression (52). Subsequent studies have determined that enhanced eNOS mRNA stability by statins is ultimately a result of polyadenylation as a consequence of Rho effects by increased RNA polymerase II activity (47). Under basal conditions, eNOS mRNA is characterized by a short 3′-poly(A) tail of <25 nt. Ribonuclease protection assays used to assess eNOS mRNA polyadenylation in bovine ECs treated with statins for up to 24 h confirm a dose- and time-dependent effect with a maximal effect at 24 h and 165 nt poly(A) tails conferring transcript stability identified at that time point.

Independent of direct effects on NO expression, statins also promote increased NO indirectly through NADPH oxidase inhibition, as would be predicted by decreased superoxide availability to react with NO to form peroxynitrite. This is

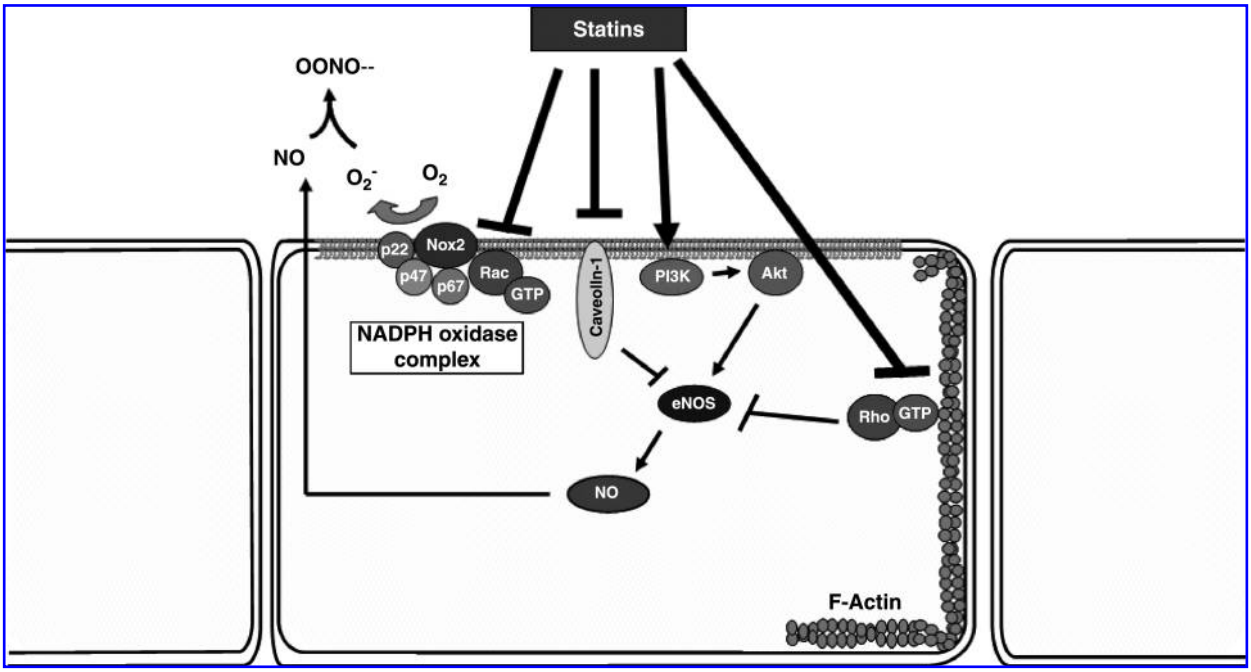

FIG. 4. Statin-induced increased NO bioavailability. Statins increase NO through multiple mechanisms. Inhibition of RhoA geranygeranylation results in increased eNOS mRNA stability due to polyadenylation through increased RNA polymerase II activity, whereas downregulation of caveolin-1 affects the disinhibition of eNOS, favoring its association with Hsp90 and subsequent activation. Separately, statins induce early activation of PI3-K/Akt, which also promotes eNOS activation, although Akt inhibition is evident later. Statin inhibition of NADPH oxidase results in decreased generation of superoxide available to react with NO to form peroxynitrite ($OONO^-$), with consequent increased available NO.

supported by evidence of increased eNOS expression and augmented NO-mediated vasodilatation in response to acetylcholine in rat aortas treated with statins compared with controls, whereas no difference is detectable by this measurement between statin-treated aortas and controls treated with superoxide dismutase, an antioxidant that catalyzes the dismutation of superoxide (83). These findings suggest that relative alterations in NO and ROS are an important determinant of vascular regulation by statins, more than the effects on either molecule alone. Direct measurements of NO and peroxynitrite by nanosensors applied to EC monolayers confirm the ability of statins to attenuate the shift in the relative abundance of these molecules in favor of peroxynitrite induced by the administration of oxidized LDL (40).

Separately, statins also induce increased eNOS activity through Akt, as mentioned earlier. The link between Akt signaling and eNOS activity was initially suggested by the observation that both are augmented in ECs subjected to shear stress. Subsequently, it was determined that inhibition of phosphatidylinositol 3-kinase (PI3-K), a signaling molecule upstream of Akt, inhibits EC shear stress–induced NO production (16). Statin treatment of ECs induces early (within 30 min) translocation of Akt to the cell membrane (74), an event requisite for subsequent eNOS activation (33). More precisely, Akt translocates to EC lamellipodia and filopodia in response to statins, and this effect is disrupted both by mevalonate and by the inhibition of PI3-K. Translocation is also inhibited on mutation of the pleckstrin homology domain of Akt, a domain that binds to lipid products downstream of PI3-K and that drives membrane targeting (8).

Statin treatment also induces tyrosine phosphorylation of the PI3-K p85 subunit (74), consistent with the initiation of signaling upstream of Akt. This results in Akt phosphorylation, which is inhibited by wortmannin, a PI3-K inhibitor, and subsequent eNOS phosphorylation, which itself is inhibited by overexpression of dominant-negative Akt (50). Interestingly, cholesterol added to the extracellular media of ECs inhibits both Akt translocation and PI-3 kinase/Akt activation induced by statin treatment (74). Given the time course of these events, this finding suggests a rapid turnover of intracellular cholesterol and further suggests the importance of cholesterol-rich microdomain (caveolin) depletion in statin-induced Akt signaling.

However, a review of the literature provides a somewhat unclear picture of differential Akt signaling in response to statins that is both dose- and time-dependent, with increased Akt phosphorylation early (within 15 min) and an inhibition of Akt phosphorylation after prolonged statin treatment (>12 h). For example, inhibition of Akt has been reported in EC treated for 48 h with 0.5 μM simvastatin (67) or cervistatin 25 ng/ml for 12–24 h (82), whereas reports of Akt activation by statins include ECs treated with simvastatin (0.5–1 μM) for 15 min to 3 h, with a peak effect at 1 h (50, 74).

Increased eNOS activation is also induced by statins through a cholesterol-dependent mechanism. As eNOS activity is negatively regulated by caveolin, the primary constituent of EC plasmalemmal microdomains that are dependent on cholesterol uptake by the cell, cholesterol depletion by statins results in decreased caveolin-1 expression and subsequent disinhibition of eNOS activation (27). Prolonged

eNOS activation is then facilitated by an augmented association with Hsp90 (12, 36). Notably, these effects of statins have been reported after extended treatment of ECs (48 h). Accordingly, the current data suggest that the sustained effects of statins on eNOS activity are due to the combined effects of increased Akt activity early and then later due to both increased eNOS mRNA stability and decreased caveolin-1 expression.

Statins and Endothelial Gene Expression

We previously reported differential effects of statins on EC gene expression (Fig. 5), including the upregulation of specific cytoskeletal regulators such as RhoA (43). We also reported statin-mediated upregulation of thrombomodulin, a glycoprotein expressed on the surface of ECs and a mediator of coagulation, fibrinolysis, and inflammation. The mechanisms underlying these effects have not been fully characterized, although, as noted earlier, it is known that some genes are differentially expressed in response to statins through effects on mRNA stability, including eNOS and the angiotensin II AT-1 receptor.

In addition to increased RhoA expression, specific effects of statins on genes involved in cytoskeletal regulation include a modest increase in Rac1 expression as well as decreased expression of Rho GDP dissociation inhibitor and increased expression of specific Rho GAPs and GEFs (43). Taken together, these effects are consistent with the consequent increased GTP loading of Rho GTPases by statins discussed previously. Importantly, increased RhoA gene expression by statins has been reported to correspond to increased protein expression as well, although we have not been able to identify changes in Rho GTPase protein expression by statins (14, 53). Nonetheless, the potential increased expression of RhoA by statins has been attributed to a negative-feedback mechanism, as inhibitors of cytoskeletal signaling downstream of RhoA including by inhibition of myosin light chain kinase or cytochalasin D also increases RhoA mRNA and protein expression (53).

In addition to thrombomodulin, a number of other genes involved in coagulation are differentially expressed in response to statins, including tissue factor (23) and plasminogen activator inhibitor-1 (PAI-1) (11). Separate reports have linked statin-induced thrombomodulin expression to inhibition of Rac1 and Cdc42 geranylgeranylation (although not RhoA) (59, 62) and to NO generation (73), but the attenuation of agonist-induced EC tissue factor and PAI-1 expression by statins has been linked to the inhibition of RhoA geranygeranylation (49). We also reported decreased expression of the thrombin receptor protease-activated receptor-1 (PAR-1) in ECs treated with simvastatin.

The functional significance of these effects of statins on these particular genes is complex but can be thought of in the context of signaling by the inflammatory mediator thrombin, a serine protease that promotes platelet activation and fibrin clot formation. Thrombin binds to thrombomodulin on the surface of ECs, and this complex then converts plasma serine protease zymogen protein C to its activated form, APC. APC in turn inactivates factors Va and VIIIa, effecting decreased thrombin formation (21). Separately, tissue factor serves as a cell-surface receptor for factor VIIa, which together drive the extrinsic coagulation pathway that culminates in thrombin generation (81). PAI-1 is a serine protease inhibitor that specifically inhibits tissue plasminogen activator and

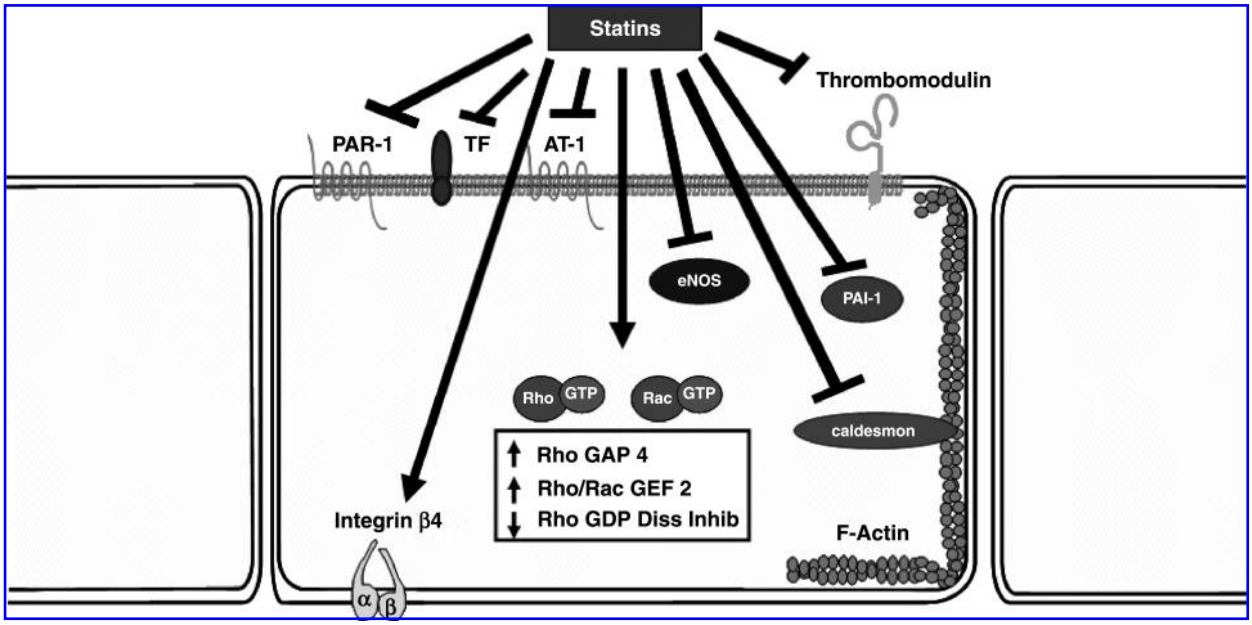

FIG. 5. Differential endothelial gene expression by statins. Statin treatment induces significant EC gene expression changes, including upregulation of the small GTPases RhoA and Rac1, with effects as well on specific mediators of RhoGTPase activation (GEFs and GAPs). In addition, statins induce differential effects on mediators of coagulation, including the upregulation of thrombomodulin and the downregulation of the thrombin receptor PAR-1, tissue factor (TF), and PAI-1. Numerous other genes relevant to endothelial function and cytoskeletal regulation are also differentially regulated by statins, including increased expression of eNOS and integrin $\beta 4$ and decreased expression of caldesmon and AT-1.

urokinase with consequent inhibition of fibrinolysis (26). Accordingly, the combined effects of increased expression of thrombomodulin and decreased expression of tissue factor and PAI-1, as well as PAR-1, would predict statin-mediated EC thrombin resistance consistent with our previous reports (14, 43).

We also identified two other EC genes of interest that are markedly differentially regulated by statins. Caldesmon, a cytoskeletal protein involved in the actin rearrangement and contractility associated with EC barrier disruption induced by a number of agonists including thrombin (10, 75), is decreased in expression more than twofold at both the gene and protein levels in response to simvastatin. Perhaps most intriguing is the dramatic upregulation of integrin β4 (more than sevenfold increase) induced by simvastatin. The integrins are heterodimeric transmembrane proteins with α and β subunits that mediate both inside-out and outside-in signaling pathways. Although eight β-subunits have been identified, integrin β4 is uniquely characterized by its long cytoplasmic tail, which comprises >1,000 amino acids (41). Although little is known about the role of integrin β4 in endothelial cells, the integrin β-subunits have been implicated in activation of the Rho GTPases as well as MAPK signaling (37), pathways highly relevant to endothelial signaling in response to inflammation and injury. Accordingly, we are now actively investigating the functional significance of integrin β4 upregulation in ECs by statins.

Clinical Implications

Taken as a whole, what we now know about the ability of statins to mediate EC signaling further supports the idea that these drugs are highly vascular protective, independent of their effects on serum cholesterol levels. In particular, EC barrier regulation by statins, predominantly through inhibition of Rho GTPase gerany geranylation, confers significant protection in the context of agonist-induced barrier disruption. Separately, increased eNOS activity by statins would predict enhanced vascular function as result of induced vasodilation, inhibition of platelet activation, and decreased expression of a number of proinflammatory genes. Direct evidence of this is provided by reports of endothelium-dependent relaxation of rat aortas effected by simvastatin through an NO-dependent mechanism (6) as well as simvastatin-mediated restoration of endothelium-dependent relaxation of aortas from rats subjected to chronic NO synthase inhibition by N^{ω}–nitro-L-arginine methyl ester (L-NAME) (68). In the latter study, rats received L-NAME (70 mg/kg/d) for 8 weeks with or without simvastatin (1 mg/kg), with all drugs discontinued 3 days before the isolation of aortas for evaluation, thus providing evidence of prolonged and sustained effects of statins in this regard.

On the basis of the diverse properties of statins on endothelial function, these drugs are now being considered as potential novel therapeutic agents for a wide range of clinical conditions. Perhaps not surprisingly, statins have shown promise in a number of animal models of disease characterized by vascular leak or dysfunction. For example, we reported the protective effects of simvastatin in a murine model of acute lung injury (ALI) (44). In these studies, LPS was administered intratracheally to induce injury, and measures were taken of lung vascular leak and inflammation including bronchoalveolar lavage, albumin, and cell counts to confirm the attenuation of injury in animals pretreated with simvastatin *via* intraperitoneal injection. Evidence of decreased tissue edema and inflammatory cells on lung histology of animals treated with simvastatin compared with injured controls further supported our findings. Notably, a significant role for vascular dysfunction and, in particular, NADPH oxidase activity in our model was previously characterized (34, 70), suggesting that protection conferred by statins in this setting is due at least in part to effects related to these specific mechanisms of injury.

The clinical literature also supports the idea that statins may have a beneficial effect on outcomes in patients predisposed to vascular leak syndromes such as ALI. In particular, hospitalized patients with pneumonia or bacteremia, populations at risk for developing sepsis and ALI, have been independently studied with respect to evidence of a beneficial effect of statins (5, 48, 58, 64, 77). These studies suggest a protective effect of statins in these clinical contexts with respect to both mortality and a variety of other clinically relevant end points. A recent meta-analysis identified 20 studies in which the effect of statins in infections and sepsis was studied (25). Of these, 11 studies had data regarding mortality as the main outcome, and eight of these reported evidence of decreased mortality associated with statin use (one study did report increased mortality, whereas the other two reported no association). Not surprisingly, at the time of this writing, a number of clinical trials are now under way to examine this question further.

One link between the *in vitro* effects of statins on the endothelium and their potential protective effects in relevant clinical settings is suggested by evidence of increased superoxide production in whole blood collected from patients with sepsis and then stimulated with phorbol myristate acetate, relative to controls (19). These effects are attenuated by simvastatin pretreatment of the whole blood, consistent with an inhibition of NADPH oxidase. Notably, however, it is likely the combined effects of statins on EC signaling that account for their novel therapeutic potential rather than any single effect, as indicated by a lack of evidence for a clinical benefit associated with the use of antioxidants in sepsis (3).

Conclusion

The interplay between seemingly diverse properties of statins on EC signaling is an important determinant of their functional consequences. The inhibition of Rho GTPases mediates actin cytoskeletal changes, with direct effects on vascular function but also mediates both NADPH oxidase activity (through Rac) and eNOS expression (through Rho), which accounts for additional vascular functional effects. At the same time, differential expression of genes involved in a variety of pathways, including cytoskeletal regulation and coagulation, have also been linked to statin Rho GTPase effects. In addition, decreased superoxide generation through NADPH oxidase inhibition itself favors increased NO bioavailability through an attenuation of NO conversion to peroxynitrite. Moreover, distinct mechanisms have also been identified contributing to these same effects, including increased Akt signaling, effecting eNOS activation early in response to statin treatment. The culmination of all of these statin-mediated events is consistent with improved vascular

function and protection in a variety of clinical contexts. However, aside from the intriguing clinical implications of the direct effects of statins on the endothelium, these drugs may also serve as a powerful tool to help explore endothelial signaling and function in general, which may ultimately lead to other novel targets and strategies for treating diseases characterized by derangements of vascular function.

Abbreviations

ALI, acute lung injury; APC, activated protein C; AT-1, angiotensin II type-1; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; GAP, GTPase-activating protein; GDI, guanine dissociation inhibitor; GEFs, guanine nucleotide exchange factors; GGP, geranygeranyl-pyrophosphate; HGF, hepatocyte growth factor; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; L-NAME, N^{ω}-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PAI-1, plasminogen activator inhibitor-1; PAR-1, protease-activated receptor-1; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; S1P, sphingosine 1-phosphate; TER, transendothelial electrical resistance.

Disclosure Statement

No competing financial interests exist.

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