Endothelial Cell Barrier Protection by Simvastatin: GTPase Regulation and NADPH Oxidase Inhibition

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Running title: Endothelial barrier regulation by simvastatin

This work acknowledges support from the NHLBI (HL 77134, HL 58064), the Parker B Francis Foundation (JRJ) and the Dr. Lowell T Coggeshall Endowment (JGNG).
Abstract

The statins, HMG CoA-reductase inhibitors which lower serum cholesterol, exhibit myriad clinical benefits including enhanced vascular integrity. One potential mechanism underlying increased endothelial cell (EC) barrier function is inhibition of geranylgeranylation, a covalent modification enabling translocation of the small GTPases Rho and Rac to the cell membrane. While RhoA inhibition attenuates actin stress fiber formation and promotes EC barrier function, Rac1 inhibition at the cell membrane potentially prevents activation of NADPH oxidase and subsequent generation of superoxides known to induce barrier disruption. We examined the relative regulatory effects of simvastatin on RhoA, Rac1 and NADPH oxidase activities in the context of human pulmonary artery EC barrier protection. Confluent EC treated with simvastatin demonstrated significantly decreased thrombin-induced FITC-dextran permeability, a reflection of vascular integrity, which was linked temporally to simvastatin-mediated actin cytoskeletal rearrangement. Compared to Rho inhibition alone (Y27632), simvastatin afforded additional protection against thrombin-mediated barrier dysfunction and attenuated LPS-induced EC permeability and superoxide generation. Statin-mediated inhibition of both Rac translocation to the cell membrane and superoxide production were attenuated by geranylgeranyl pyrophosphate indicating that these effects are due to geranylgeranylation inhibition. Finally, thrombin-induced EC permeability was modestly attenuated by reduced Rac1 expression (siRNA) whereas these effects were made more pronounced by simvastatin pretreatment. Together, these data suggest EC barrier protection by simvastatin is due to dual inhibitory effects on RhoA and Rac1 as well as the
attenuation of superoxide generation by EC NADPH oxidase and contribute to the molecular mechanistic understanding of the modulation of EC barrier properties by simvastatin.

**Keywords:** Rho, Rac, cytoskeleton, permeability, statins
Introduction

The statins, a class of HMG-CoA reductase inhibitors, are widely used for their ability to significantly lower serum cholesterol levels and their beneficial effects on morbidity and mortality associated with coronary artery disease. Recently, however, there has been intense interest in the precise mechanism of action of this class of drugs as the clinical effects of statins are now recognized to extend well beyond their lipid-lowering properties (2, 4). In this regard, we have previously reported that endothelial cell (EC) barrier protection is conferred by simvastatin following prolonged treatment (16 h) as measured by transendothelial electrical resistance (TER) (21). Subsequently, we employed a murine model of acute inflammatory lung injury (ALI) and confirmed a marked attenuation of indices of inflammation and vascular leak by simvastatin consistent with direct vascular-protective effects (22). While the potential clinical implications of these findings are significant, the mechanisms underlying EC barrier regulation by simvastatin remain poorly characterized.

Statin inhibition of HMG-CoA reductase attenuates cholesterol synthesis via the inhibition of the prenylation pathway, a necessary series of covalent modifications of cholesterol precursors which culminates in either farnesylation or geranylgeranylation and the addition of either a 15- or 20-carbon side chain, respectively (46). Although cholesterol is one product of prenylation, this same pathway also promotes the activation of small GTPases such as Rho and Rac via
their geranylgeranylation and subsequent translocation to the cell membrane (16, 34). This localization of small GTPases to the cell membrane in turn favors GTP binding and thus GTPase activation. The relevance of these effects to EC barrier function is significant as RhoA activation is an important mediator of acto-myosin contraction, actin stress fiber formation, and the resultant increased intracellular tensile forces that directly determine EC barrier integrity (7). Additionally, the inhibition of RhoA by statins leads to the upregulation of endothelial nitric oxide synthase (eNOS) and, subsequently, increased nitric oxide (NO) bioavailability which promotes vasorelaxation and inhibits platelet activation (25).

The role of Rac in EC barrier function is less well characterized. We have previously reported that Rac1 activation is associated with the translocation of cortactin, an actin-binding protein, to the cell periphery and the subsequent polymerization of cortical actin, key elements involved in the augmentation of EC barrier function (8). A complementary role for Rac in EC barrier regulation is its requirement at the cell membrane as a necessary component of the NADPH oxidase complex which drives superoxide generation, a key component of reactive oxygen species which are known to be EC barrier-disruptive (19, 29, 37). Although the recognized inhibition of the prenylation pathway by statins would predict the subsequent inhibition of both Rho and Rac, we previously reported that prolonged simvastatin treatment (16 h) results in an increase in total cell Rac1-GTP content, consistent with the paradoxical activation of Rac1. While the functional significance of this increase in Rac1-GTP
loading is unclear, this finding is not inconsistent with the localized inhibition of Rac1 at the cell periphery via effects on geranylgeranylation.

Based upon our current studies, we now hypothesize a dual effect of simvastatin on Rac1 activation which is dependent on Rac1 localization within the cell. Rac1 inhibition at the cell periphery (i.e. membrane) contributes to EC barrier protection via the inhibition of NADPH oxidase and superoxide generation whereas activation of cytosolic Rac1 independently promotes EC barrier function via direct effects on the EC cytoskeleton. We have examined the relative contributions of both RhoA and Rac1 in simvastatin-mediated EC barrier protection and have characterized the effects of simvastatin on Rac-dependent NADPH oxidase activity as it relates to EC barrier function. These results provide further understanding of the mechanisms underlying the vascular-protective effects of statins and may potentially lead to new clinical applications for this class of drugs.

Some of the results of these studies have been previously reported in the form of an abstract at the 2007 International Meeting of the American Thoracic Society (5).
Materials and Methods

Materials and reagents: Antibodies for the NADPH oxidase subunit p47phox and Rac1 were purchased from Upstate (Charlottesville, VA). Simvastatin was provided by Merck (Whitehouse Station, NJ) and siRNA used as control was purchased from Dharmacon (Layfayette, CO). Specific siRNA for human Rac1 and siPORT™ Amine transfection agent were obtained from Ambion (Austin, TX). Dihydroethidium (DHE) was obtained from Molecular Probes (Eugene, OR). The pharmacologic Rho kinase inhibitor, Y27632 was purchased from Calbiochem (San Diego, CA). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Cell culture: Human pulmonary artery EC were purchased from Clonetics (San Diego, CA) and were cultured in EGM-2 supplemented with 2% FBS, hydrocortisone, hFGF, VEGF, ascorbic acid, hEGF, GA-1000, Heparin, R3-IGF-1 (Clonetics). The cells were incubated in 75 cm² flask and cultured at 37ºC in 5% CO₂ and 95% air. All cells were used at passages 4-8.

Silencing RNA: The siRac1 (Ambion) target sequence is 5’-GGAGAUUGGGUGCGUAAAA-3’. Human Cdc42 siRNA was purchased from Dharmacon. A scramble siRNA was used as control (Dharmacon) with sequence 5’-UAGCGACUAAACACAUCAA-3’. EC were plated on 6 wells plate (60-80% confluent) or in a transwell permeability insert (1x10⁵/insert) 24 hours
prior to transfection with siRNA using siPORT™ Amine (Ambion). Briefly, the cells were incubated by OPTI-MEM for 20 min at 37°C. siRNA and siPORT™ Amine were each mixed with OPTI-MEM (Invitrogen, Carlsbad, CA) for 10 min at room temperature. Equal volumes of each solution were mixed together for another 10 min at room temperature. After removal of the OPTI-MEM, the final mixture was mixed with EGM-2 medium (Clonetics) and added to cells. After overnight incubation, the transfection medium was aspirated and replaced with complete medium and incubated for another 48-72 h.

**Measurement of superoxide production:** DHE, a cellular membrane-permeable fluorophore hydroethidine, was used to measure EC superoxide production. Briefly, EC were serum starved overnight and then loaded with 10 µM DHE in basal EGM-2 medium (Clonetics) for 30 min. After washing with PBS, the cells were then stimulated with lipopolysaccharide (LPS) for 1 h in basal medium without supplements. An aliquot (100 µl) of medium was used for fluorescent density assay on an Aminco-Bowman series 2 spectrofluorometer (Thermo Electron Corporation, Brookfield, WI) with excitation and emission set at 470 and 610 nm, respectively.

**Membrane isolation:** EC were grown to confluence, washed with ice-cold PBS and harvested with hypotonic buffer (5 mM Tris-HCl, pH 7.6, 2mM EDTA containing proteinease inhibitor, 1 mM NaVO₄ and 20 mM NaF). Detached cells were homogenized twice for 30 s each. After centrifugation at 400 g for 10 min at
4°C, the supernatant was then centrifuged at 12000 g for 30 min. The pellet was washed three times with HEPES-Tyrode’s buffer containing proteinase inhibitors. To dissolve the membrane protein, the same buffer containing 1% (v/v) Nonidet P-40, 4 mM CHAPS, or 10 mM CHAPS, 0.5% (w/v) deoxycholate was added to the pellet for 60 min at 4°C and the centrifuged at 14,000 g for 30 min at 4°C. The supernatant was collected for protein assays.

**Western blotting:** Samples were mixed with Laemmli sample buffer, boiled and subsequently analyzed by SDS-PAGE as per standard protocol. After transfer to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), Western blotting was performed using appropriate primary antibodies. The membranes were blotted with horseradish peroxidase-conjugated secondary antibody prior to visualization via chemiluminescence (Amersham Biosciences, Piscataway, NJ). Blot density was determined by Alpha Imager software (Alpha Innotech, San Leandro, CA).

**In vitro vascular permeability assay:** A commercially available kit (Chemicon, Temecula, CA) was used to measure EC monolayer permeability to high molecular weight proteins utilizing 2,000 kDa FITC-dextran based upon the transwell model we previously described (13). Briefly, a transwell insert was coated with collagen for 1 h at room temperature and EC then seeded at a density of 1× 10^5/well in a final volume of 400 µl EGM-2 with supplements
(Clonetics). The inserts were placed into 24 wells plates containing 500 µl medium for overnight. To measure agonist-induced EC permeability, 100 µl FITC-dextran was added into the insert and incubated for 1 h. The insert was then removed and 100 µl medium collected from the bottom chamber. The fluorescent density of samples was analyzed on a Titertek Fluoroskan II Microplate Fluorometer (Diversified Equipment Company, Lorton, VA) at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

**Immunofluorescent microscopy:** Confluent EC grown on coverslips were exposed to experimental conditions, fixed with 3.7% formaldehyde, and permeabilized with 0.25% Triton X-100. After blocking with 2% BSA, F-actin was detected by staining with Texas Red–conjugated phalloidin. Cells were imaged using a Nikon video-imaging system.

**Statistical analysis:** Student’s t-test was used to compare the means of data from two different experimental groups. Significant differences between groups (p<0.05) were confirmed by ANOVA and post-hoc multiple comparisons tests. Results are expressed as the mean ± standard error.
Results

Time course of EC barrier protection and cytoskeletal rearrangement produced by simvastatin. We previously reported a delayed effect of simvastatin on EC barrier protection as measured by TER with a requirement for 16 h pretreatment to produce significant TER changes, a time frame corresponding with increased Rac1 activation (Rac-GTP). Accordingly, we have postulated that EC barrier enhancement by simvastatin largely involves activation of Rac. We sought to further characterize the time-dependent effects of simvastatin using a FITC-dextran transwell assay to measure EC monolayer permeability (15). EC grown to confluence on transwell filters were stimulated with thrombin (1 U/mL, 1 h) resulting in significant increases in passage of FITC-dextran across the monolayer that was significantly attenuated by simvastatin pretreatment (5 µM) as brief as 6 hrs with a ~60% reduction in permeability compared to control (Figure 1A). Of note, a significant effect was not appreciable after 2 h of simvastatin treatment prior to thrombin stimulation and in separate experiments we were unable to detect an effect with either simultaneous simvastatin treatment or simvastatin administered at any time after thrombin (data not shown). However, we observed a significant effect of simvastatin on the EC actin cytoskeleton consistent with evidence of early EC barrier protection by simvastatin (Figure 1B). After only 2 h of simvastatin treatment (5 µM), EC monolayers demonstrated fewer paracellular gaps, enhanced cortical actin, and decreased transcellular stress fibers compared to unstimulated control cells, changes that were more pronounced at 16 h of pretreatment.
EC barrier protection by simvastatin is not attributable to RhoA inhibition alone. To characterize the relative functional effect of RhoA inhibition by simvastatin, thrombin-induced EC permeability was measured via FITC-dextran translocation across EC monolayers grown to confluence in transwell plates prior to pretreatment with Y27632 (10 µM, 30 min), a pharmacologic Rho kinase inhibitor, at concentrations that would produce nearly complete Rho inhibition (20). While Rho kinase inhibition produced substantial attenuation of thrombin-induced EC barrier disruption (~80% inhibition), simvastatin pretreatment (5 µM, 16 h) significantly augmented this effect producing complete attenuation (Figure 2). These data suggest that RhoA inhibition contributes but does not wholly account for the marked EC barrier-protective effects of simvastatin.

Simvastatin attenuates LPS-induced EC permeability. In addition to thrombin, we assessed the efficacy of simvastatin in a second model of agonist-mediated vascular permeability utilizing the bacterial endotoxin, LPS. In EC monolayers grown to confluence in transwell plates, LPS treatment (1 µg/ml, 1.5 h) increased FITC-dextran translocation consistent with enhanced permeability (Figure 3A). Similar results were also obtained using a lower dose of LPS for a longer duration (100 ng/ml, 6 h, data not shown). Consistent with the effects observed in thrombin-stimulated EC, simvastatin pretreatment (5 µM, 16 h) abrogated LPS-induced (1 µg/ml, 1.5 h) EC barrier disruption (LPS added 14.5 h after simvastatin). EC barrier protection by simvastatin, however, was inhibited by simultaneous treatment with
geranylgeranyl pyrophosphate (GGPP, 10 µM, 16 h) indicating that these effects are due to the inhibition of geranylgeranylation. Finally, evidence of the inhibition of LPS-induced EC barrier disruption by simvastatin is further supported by immunofluorescence imaging (Figure 3B) as LPS-induced (100 ng/ml, 1.5 h) actin stress fiber formation and paracellular gaps were abrogated by simvastatin pretreatment (5 µM, 16 h).

Simvastatin inhibits membrane localization of Rac1 and p47phox and attenuates LPS-induced superoxide generation. As the inhibition of geranylgeranylation by simvastatin would predict diminished Rac localization to the cell membrane, we examined the cellular distribution of Rac and p47phox, regulatory subunits of the NADPH oxidase complex (27), in response to simvastatin. Simvastatin pretreatment (5 µM, 16 h) effected a marked decrease in the amount of EC membrane Rac1 concomitant with a pronounced increased in cytosolic Rac1, effects that were independent of LPS treatment (Figure 4A). In addition, p47phox levels were similarly redistributed in response to simvastatin (decreased at the membrane and increased in the cytosol), again independent of LPS treatment (Figure 4B). These results are consistent with an inhibition of NADPH oxidase assembly by simvastatin as the organization of the regulatory subunits at the cell membrane is required for complex activation.

As NADPH oxidase activation requires Rac, we next investigated the effects of simvastatin on LPS-induced superoxide generation as measured by DHE
fluorescence. LPS (100 ng/ml, 6 h) increased superoxide generation (~1.5 fold) as compared to control cells (Figure 4C). These effects were significantly blunted, however, by simvastatin (5 µM, 16 h) pretreatment (~80% decrease compared to LPS alone). By comparison, superoxide dismutase (SOD, 150 U/ml, 6 h) effected a similar decrease in LPS-induced superoxide generation. Moreover, similar to the effects of simvastatin on LPS-induced EC permeability, LPS-induced superoxide production in simvastatin pretreated EC was indistinguishable from that of unstimulated control cells. Notably, LPS-induced ROS generation was not associated with increased Rac1 or p47^phox^ translocation to the cell membrane suggesting that these events are necessary but not sufficient for membrane NADPH oxidase activity. Moreover, while simvastatin treatment resulted in both Rac1 and p47^phox^ translocation from the membrane to the cytosol, basal ROS as measured by DHE fluorescence was not effected. This may be consistent with ROS generated from Nox4-based oxidase which is constitutively active and does not rely on p47^phox^ or other regulatory subunits for activation (30).

Finally, we sought to determine if the inhibition of LPS-induced superoxide generation by simvastatin could contribute to its barrier protective effects. In subsequent experiments, concomitant treatment of EC with xanthine and xanthine oxidase (200 µM and 30 mU/ml, respectively, 1 h) to generate superoxide resulted in significant EC barrier dysfunction as measured by FITC-dextran flux (Figure 4D). These data support the idea that, independent of effects on cytoskeletal
rearrangement, the inhibition of superoxide generation is a distinct mechanism of simvastatin-mediated EC barrier protection.

**Simvastatin effects on superoxide generation and Rac1 translocation are due to geranylgeranylation inhibition.** To establish the role of the inhibition of Rac1 geranylgeranylation by simvastatin in its attenuation of superoxide generation, GGPP was used to override this effect. Consistent with prior experiments, simvastatin pretreatment (5 μM, 16 h) significantly attenuated LPS-induced (100 ng/ml, 6 h) superoxide generation (Figure 5A). This effect was even more pronounced than that observed with diphenyleneiodonium (DPI), an inhibitor of flavoenzymes including NADPH oxidase (31). However, inhibition of superoxide was no longer significant when cells were concomitantly treated with simvastatin (5 μM, 16 h) and GGPP (10 μM, 16 h). Moreover, GGPP attenuated the cellular redistribution of Rac1 by simvastatin (5 μM, 16 h) (Figure 5B). Collectively these results suggest that these findings are attributable to the inhibition of geranylgeranylation by simvastatin rather than any potential non-specific effects. Importantly, simvastatin did not affect a significant change in total Rac as measured by the combined densitometry of membrane and cell fractions from these experiments or in whole cell lysates in separate experiments (data not shown). Although we have previously reported modest upregulation of Rac1 gene expression (~1.3 fold increase) by simvastatin (3), our findings now suggest that this does not correspond to significant transcriptional upregulation of Rac1. Finally, similar to the
effects of simvastatin (5 µM, 16 h), siRNA specific for Rac1 also effected a significant attenuation of LPS-induced (1 µM, 30 min) superoxide generation (Figure 5C).

**EC barrier protection by simvastatin is not solely attributable to Rac1 inhibition.** We again employed siRNA specific for Rac1 to assess the contribution of Rac1 inhibition on agonist-induced EC barrier permeability relative to the effects of simvastatin pretreatment. Using EC transfected with scrambled siRNA as controls, siRac1 effected a ~20% decrease in thrombin-induced (1 U/ml, 1 h) EC permeability as measured by FITC-dextran transwell permeability (Figure 6). However, a more pronounced effect was observed in EC that were both transfected with siRac1 and pretreated with simvastatin (5 µM, 16 h). This effect was not significantly different from simvastatin pretreatment alone prior to thrombin stimulation. These data suggest that the inhibition of Rac1 and NADPH oxidase activity, similar to RhoA inhibition, contributes to but is not solely responsible for EC barrier protection by simvastatin.

**EC barrier protection by simvastatin is not mediated by inhibition of Cdc42.** As the Rho GTPase known as Cdc42 is dependent on geranylgeranylation and may also regulated EC permeability, we used siRNA to determine its functional role in simvastatin-mediated EC barrier protection. Compared to controls, Cdc42 silencing did not appreciably effect either thrombin-induced (1 U/ml, 1 h) EC permeability or the degree of protection conferred by simvastatin pretreatment (5 µM, 16 h) as
measured by transmonolayer dextran flux (Figure 7). Thus, these data fail to support a significant role for Cdc42 in simvastatin-mediated EC barrier regulation.
Discussion

The ability of the statins to promote EC barrier integrity and directly enhance vascular function is now well recognized with multiple potential mechanisms identified (3, 21, 32, 40, 41, 45). We previously reported novel lung vascular protection conferred by simvastatin in a murine model of ALI (22), results subsequently validated by other reports (11, 44). However, the relative functional significance of the direct effects of simvastatin on the endothelium in this setting is unknown. Our data now suggest that inhibition of membrane activation of the small GTPases, RhoA and Rac1, are important determinants of EC barrier protection by simvastatin. We have now identified dual effects by simvastatin on Rac1 activation that are dependent on Rac1 cellular localization with Rac1 inhibition occurring at the cell membrane but increased Rac1 activation (Rac1-GTP) in the cytosol (21). Notably, inhibition of geranylgeranylation by simvastatin would also predict inhibitory effects on Cdc42, a Rho family GTPase associated with both EC cytoskeletal regulation and potential barrier-regulatory effects (26, 27). While there are reports suggesting a role for Cdc42 in EC barrier regulation and permeability (42), we found no evidence that Cdc42 is involved in either thrombin-induced EC barrier disruption or the protective effects of simvastatin in this setting.

We propose two specific mechanisms of EC barrier regulation by simvastatin that contribute to its vascular-protective effects: dynamic cytoskeletal rearrangement and the inhibition of NADPH oxidase activity and subsequent superoxide generation (21,
Consistent with our prior reports, EC cytoskeletal rearrangement by simvastatin is characterized by a reduction in transcellular actin stress fibers, indicative of RhoA inhibition, as well as increases in peripheral polymerized actin. This latter effect is associated with Rac1 activation in response to other EC barrier-protective agonists we have studied which produce similar changes in cytoskeletal rearrangement including sphingosine 1-phosphate, hepatocyte growth factor and ATP (14, 23, 28). Consistent with early simvastatin effects on Rho GTPase activity, we now observed evidence of similar cytoskeletal changes in simvastatin-treated EC within 2 h that were more pronounced at 16 h. These changes correspond to the time-dependent effects of simvastatin on EC barrier protection as measured by transmonolayer FITC-dextran permeability. Relying solely on measurements of transendothelial electrical resistance, we were previously unable to detect evidence of EC barrier protection by simvastatin with pretreatment durations of less than 16 h and had hypothesized this was likely due to delayed Rac1 activation (21). Although these measurements are thought to be fairly sensitive they are at least somewhat limited by virtue of being an indirect measurement of permeability. Indeed, our data now draw into question the sensitivity of our earlier findings and require a revision of our original hypothesis as evidence of early barrier protection (dextran flux) by simvastatin clearly indicates a barrier-regulatory mechanism independent of late Rac1 activation. However, the additional benefit of simvastatin above the inhibitory effects on RhoA suggests a Rho-independent protective mechanism. In this regard, our data are consistent with the contribution of membrane-associated Rac1 inhibition with downstream consequences for NADPH oxidase activity.
Activation of NADPH oxidase has been implicated as an important mediator of injury in various models of murine ALI (12, 35). Separately, simvastatin has been shown to inhibit phorbol ester-induced superoxide production in whole blood from patients with sepsis (10). Moreover, in a model of diabetes-induced vascular injury mediated by oxidative stress, the selective inhibition of Rac1 has been found to be protective (38). Our data indicate that simvastatin produces a marked decrease in LPS-induced superoxide production consistent with the inhibition of NADPH oxidase activity. These effects were associated with reduced translocation to the cell membrane of the NADPH oxidase regulatory subunits, Rac1 and p47phox via inhibition of geranylgeranylation and an attenuation of LPS-induced EC permeability. We observed a modest effect on thrombin-induced EC permeability using siRac1 suggesting that Rac1 inhibition alone does not represent the primary mechanism of simvastatin-mediated EC barrier protection. The increased cytosolic Rac1-GTP induced by statins potentially represents a significant mediator of EC barrier protection in which case the use of siRac1 would not be expected to replicate barrier protection comparable to statins. Notably, we used two different agonists in our current studies, thrombin and LPS. While these agonists evoke different signaling pathways culminating in EC barrier disruption they do share some common features including the activation of both RhoA and Rac1 (17, 39, 43) as well as the induction of EC superoxide generation (18, 27, 33). Importantly, our data suggest that the observed effects of simvastatin on EC barrier regulation are related to these events rather than to other potentially non-specific effects. Finally, our conclusion that the
protective effects of simvastatin *in vivo* are not solely due to Rac1 inhibition and the inhibition of superoxide generation via NADPH oxidase is indirectly supported by the lack of any clinical evidence of a therapeutic role for the use of antioxidants in ALI (1).

Aside from EC actin cytoskeletal effects, statin inhibition of RhoA also effects eNOS upregulation which leads to increased NO production (25). In turn, NO serves as a potent vasodilator as well as an inhibitor of platelet activation. Additionally, NO rapidly reacts with superoxide to produce peroxynitritirite (ONOO⁻), a powerful oxidant, the activity of which has been associated with severity of injury in human ALI (24). Undoubtedly, these effects contribute to the well described beneficial effects of statins on vascular function in a number of disparate clinical settings (15, 26, 36). However, we have previously confirmed that the attenuation of agonist-induced EC permeability by simvastatin is unaffected by concomitant treatment with L-NAME, an eNOS inhibitor (21). Consequently, we have hypothesized that cytoskeletal rearrangement alone accounts for the contribution of EC barrier protection conferred by RhoA inhibition in our experiments and are most relevant to the protective effects of simvastatin in our murine model of ALI.

The limitations of our current study include the reliance on the use pharmacologic inhibitors in several experiments from which our conclusions are drawn. We recognize the potential non-specific effects associated with these inhibitors and acknowledge that these experiments, by themselves, are not definitive. While the
results from these experiments and the conclusions drawn are strengthened by complementary studies in which we employed siRNA, a more specific technique, the results of our study do not preclude the possibility of alternate mechanisms contributing to EC barrier protection by simvastatin.

In summary, our results confirm a dual effect of simvastatin on Rac1 with Rac1 inhibition at the cell periphery (predicted by the inhibition of geranylgeranylation) but with increased Rac1 activation intracellularly (cytosol), possibly due to the differential expression of specific regulatory genes (21). These findings implicate an important functional difference of Rac1 that is dependent on its cellular localization. While our data suggests that peripheral Rac1 inhibition corresponds to the inhibition of NADPH oxidase activity, efforts to identify the functional significance of increased intracellular Rac1-GTP induced by simvastatin are ongoing. Moreover, reports exist that statins paradoxically increase not only Rac1-GTP but RhoA-GTP as well (6, 9). Ultimately, as the Rho family GTPases are central to a variety of EC signaling pathways, a more complete understanding of the complex effects of statins on their activation may not only hold significant clinical implications but may provide highly novel insights into EC function in general.
Acknowledgements

This work acknowledges support from the NHLBI (HL 71411, HL 58064) and support from the Parker B Francis Foundation (JRJ) and the Dr. Lowell T Coggeshall Endowment (JGNG).
References


Figure Legends

Figure 1. EC barrier protection and cytoskeletal rearrangement by simvastatin.
(A) EC grown on transwell filters were stimulated with thrombin (1 U/ml, 1 h) prior to measurements of FITC-dextran permeability. Pretreatment with simvastatin (5 µM) confers time-dependent protection with a significant decrease in thrombin-induced permeability evident within 6 h (*p<0.05) and more pronounced at 16 h (**p<0.05, n = 3 for each condition). (B) Immunofluorescent imaging of confluent EC monolayers demonstrates evidence of early cytoskeletal rearrangement after treatment with simvastatin alone (5 µM) characterized by decreased transcellular actin stress fibers and enhanced peripheral actin evident as early as 2 h. These effects are associated with a dramatic decrease in the number of appreciable paracellular gap and are even more pronounced at 16 h.

Figure 2. Role of Rho inhibition in EC barrier protection by simvastatin.
Thrombin-induced (1 U/ml, 1 h) FITC-dextran translocation across EC monolayers was measured in control cells as well as subsequent to pretreatment with either simvastatin (5 µM, 16 h) or a pharmacologic Rho kinase inhibitor, Y27632 (10 µM, 30 min), or a combination of both (n = 3 for each condition). Y27632 effects a marked decrease in thrombin-induced EC permeability that is significantly augmented with the co-administration of simvastatin (*p<0.05). In addition, the protection conferred by simvastatin alone is significantly augmented by Y27632.
Basal permeability did not significantly differ amongst EC monolayers treated with simvastatin, Y27632, or the combination of both.

**Figure 3. Effect of simvastatin on LPS-induced EC permeability and cytoskeletal rearrangement.** (A) Compared to untreated controls, EC monolayer permeability is significantly increased in response to LPS (1 µg/ml, 1.5 h) as measured by FITC-dextran translocation (*p<0.05). Independently, simvastatin pretreatment (5 µM, 16h) and Rho kinase inhibition (Y27632, 10 mM, 30 min) both significantly attenuated LPS-induced (1 µg/ml, 1.5 h) EC barrier disruption (**p<0.05 and †p<0.05) (n = 3 for each condition). EC barrier protection by simvastatin was abrogated by concomitant treatment with GGPP (10 µM, 16 h). (B) EC monolayer protection by simvastatin corresponds to early evidence of decreased LPS-induced (1 µg/ml, 1.5h) paracellular gaps by immunofluorescence (white arrows) in simvastatin-treated EC (5 µM, 16 h).

**Figure 4. Rac and p47phox localization in response to simvastatin and effect of simvastatin on superoxide-induced EC barrier dysfunction.** (A) Simvastatin pretreatment (5 µM, 16 h) induces a marked decrease in EC membrane Rac1 content concomitant with a pronounced increase in cytosolic Rac1, effects that are independent of LPS treatment (*p<0.05). (B) p47phox levels are similarly redistributed in response to simvastatin (decreased at the membrane and increased in the cytosol), again independent of LPS treatment (*p<0.05). (C) Compared to control cells, superoxide generation in LPS-treated (100 ng/ml, 6 h) EC is associated with a
marked increase in superoxide production as measured by DHE fluorescence. Comparable to the effects observed with SOD (150 U/ml, 6 h), LPS-induced superoxide production is significantly attenuated by simvastatin (5 µM, 16 h) pretreatment (*p<0.05). (D) Superoxide-induced EC barrier disruption by concomitant treatment with xanthine (X, 200 µM, 1 h) and xanthine oxidase (XO, 30 mU/ml, 1 h) was significantly increased compared to controls as measured by FITC-dextran monolayer permeability (*p<0.05). (n = 3 for each condition)

**Figure 5. Role of geranylgeranylation inhibition in simvastatin-mediated EC effects.** (A) Comparable to the effects of DPI (10 µM, 16 h), simvastatin pretreatment (5 µM, 16 h) significantly attenuates LPS-induced (100 ng/ml, 6 h) superoxide generation (*p<0.05 and **p<0.05). However, compared to untreated EC, there is not a significant difference when cells are concomitantly treated with simvastatin (5 µM, 16 h) and GGPP (10 µM, 16 h). (B) GGPP (10 µM, 16 h) attenuates the cellular redistribution of Rac1 induced by simvastatin (5 µM, 16 h) as measured by densitometry of Western blots (*p<0.05). (C) The attenuation of LPS-induced (100 ng/ml, 6 h) superoxide generation by simvastatin (5 µM, 16 h) is comparable to effects of siRNA specific for Rac (*p<0.05 and **p<0.05). (n = 3 for each experimental condition).

**Figure 6. Role of Rac1 in EC barrier protection by simvastatin.** Compared to the protective effects of simvastatin (5 µM, 16 h) in EC transfected with scramble siRNA (nsRNA), thrombin-induced (1 U/ml, 1 h) EC permeability as measured by FITC-
dextran permeability is only modestly decreased via siRac1 transfection (*p<0.05 and **p<0.05). A more pronounced effect is observed in EC that are both transfected with siRac1 and pretreated with simvastatin (5 µM, 16 h) (†p<0.05, n = 3 for each experimental condition).

Figure 7. Role of Cdc42 in EC barrier protection by simvastatin. Compared to control EC transfected with scramble siRNA (nsRNA), thrombin-induced (1 U/ml, 1 h) EC permeability as measured by FITC-dextran permeability is not affected via siCdc42 transfection (*p<0.05). Moreover, compared to control EC pretreated with simvastatin no additional effect is noted in EC that are both transfected with siCdc42 and pretreated with simvastatin (5 µM, 16 h) (**p<0.05, n = 3 for each experimental condition).
Figure 1.

A. 

FITC-dextran fluorescence (arbitrary units)

- control
- thrombin
- 2 hr
- 2 hr + thrombin
- 6 hr
- 6 hr + thrombin
- 16 hr
- 16 hr + thrombin

B. 

- F-actin
- vehicle
- 2 h
- 16 h
- simvastatin
Figure 2.
Figure 3.

A. FITC-dextran fluorescence (arbitrary units)

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<td>Y27632</td>
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<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>simva</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GGPP</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B. control LPS

F-actin

**

*
Figure 4.

A. Membrane and cytosolic p47phox levels in control and simvastatin-treated cells.

B. Membrane and cytosolic p47phox levels in control and simvastatin-treated cells.

C. DHE fluorescence levels in control, simvastatin (simva), and SOD-treated cells.

D. FITC-dextran fluorescence levels in control, X, XO, and X+XO.
Figure 5.

A.  

![Graph showing DHE fluorescence levels for control, DPI, simva, and simva + GGPP treatments under control and LPS conditions.](image)

B.  

![Western blot for cytosolic and membrane Rac proteins under simvastatin and GGPP conditions.](image)

C.  

![Western blot for Rac1 and β-actin proteins under nsRNA and siRac conditions.](image)
Figure 6.

Table showing the effects of nsRNA (100nM, 72 h), siRac (100nM, 72 h), and simva (5µM, 16 h) on Rac1 activation.

**Figure showing FITC-dextran fluorescence (arbitrary units) with significance markers.**
Figure 7.

nsRNA (100nM, 72 h)            +     +     --
siCdc42 (100nM, 72 h)           -     -     +     +
simva (5µM, 16 h)               -     +     -     +

FITC-dextran fluorescence        (arbitrary units)

thrombin                         -     +     -     +
simva                             -     -     +     +

Cdc42 nsRNA (100nM, 72 h)         +     +     --
siCdc42 (100nM, 72 h)            -     -     +     +
simva (5µM, 16 h)                -     +     -     +