Endothelial cell barrier protection by simvastatin: GTPase regulation and NADPH oxidase inhibition

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Chen W, Pendyala S, Natarajan V, Garcia JG, Jacobson JR. Endothelial cell barrier protection by simvastatin: GTPase regulation and NADPH oxidase inhibition. Am J Physiol Lung Cell Mol Physiol 295: L575–L583, 2008. First published July 25, 2008; doi:10.1152/ajplung.00428.2007.—The statins, hydroxy-3-methylglutaryl-CoA reductase inhibitors that 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 activates 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 with Rho inhibition alone (Y-27632), 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 (GGPP), indicating that these effects are due to geranylgeranylation inhibition. Finally, thrombin-induced EC permeability was modestly attenuated by reduced Rac1 expression (small interfering RNA), 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.

Rho; Rac; cytoskeleton; permeability; statins

THE STATINS. A CLASS of hydroxy-3-methylglutaryl (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 because the clinical effects of statins are now recognized to extend well beyond their lipid-lowering properties (2, 4). In this regard, our laboratory has 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 indexes of inflammation and vascular leak by simvastatin consistent with direct vascular-protective effects (22). Although 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 that 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 because RhoA activation is an important mediator of actomyosin 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. Our laboratory has 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 cortactin, 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 that drives superoxide generation, a key component of reactive oxygen species that are known to be EC barrier-disruptive (19, 29, 37). Although the recognized inhibition of the prenylation pathway by statins has predicted 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 (21). Whereas 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.

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Based on our present studies, we now hypothesize a dual effect of simvastatin on Rac1 activation that 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.

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 small interfering RNA (siRNA) used as control was purchased from Dharmacon (Lafayette, CO). Specific siRNA for human Rac1 (siRac1) and siPORT Amine transfection agent were obtained from Ambion (Austin, TX). Dihydroethidium (DHE) was obtained from Molecular Probes (Eugene, OR). The pharmacological Rho kinase inhibitor, Y-27632 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 endothelial growth media-2 (EGM-2) supplemented with 2% FBS, hydrocortisone, human FGF, VEGF, ascorbic acid, human EGF, GA-1000, heparin, and R²-IGF-1 (Clonetics). The cells were incubated in 75-cm² flask and cultured at 37°C in 5% CO₂ and 95% air. All cells were used at passages 4–8.

Silencing RNA. The siRac1 (Ambion) target sequence is 5’-GGA-GAUUGGUGCUGUGAAA-3’. Human Cdc42 siRNA was purchased from Dharmacon. A nonspecific siRNA (nsRNA) was used as control (Dharmacon) with sequence 5’-UAGCGACUAAACACAUCAA-3’. EC were plated on six-well plate (60–80% confluent) or in a Transwell permeability insert (1 × 10⁵/insert) before 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) for 30 min. After being washed 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, Brookfield, WI) with excitation and emission set at 470 and 610 nm, respectively.

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 being washed 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, 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, 2 mM EDTA containing protease 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 12,000 g for 30 min. The pellet was washed three times with HEPES-Tyrode buffer containing protease inhibitors. To dissolve the membrane protein, the same buffer containing 1% (vol/vol) Nonidet P-40, 4 mM 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), or 10 mM CHAPS, 0.5% (wt/vol) 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 before visualization via chemiluminescence (Amersham Biosciences, Piscataway, NJ). Blot density was determined by Alpha Imagier 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 on the Transwell model our laboratory previously described (13). Briefly, a Transwell insert was coated with collagen for 1 h at room temperature, and EC was then seeded at a density of 1 × 10⁵/well in a final volume of 400 μl EGM-2 with supplements (Clonetics). The inserts were placed into 24-well 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 Titerlek Fluoroskan II Microplate Fluorometer (Diversified Equipment, 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 means ± SE.

**RESULTS**

**Time course of EC barrier protection and cytoskeletal re-arrangement 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) (21). 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 h with a ~60% reduction in permeability compared with control (Fig. 1A). Of note, a significant effect was not appreciable after 2 h of simvastatin treatment before 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 (Fig. 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 with 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 before pretreatment with Y-27632 (10 μM, 30 min), a pharmacological 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 (Fig. 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 (Fig. 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 (Fig. 3B) because 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. Because it would be predicted that the inhibition of geranylgeranylation by simvastatin would diminish 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 increase in cytosolic Rac1, effects that were independent of LPS treatment (Fig. 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 (Fig. 4B). These results are consistent with an inhibition of NADPH oxidase assembly by simvastatin because the organization of the regulatory subunits at the cell membrane is required for complex activation.

Because 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) compared with control cells (Fig. 4C). These effects were significantly blunted, however, by simvastatin (5 μM, 16 h) pretreatment (~80% decrease compared with LPS alone). By comparison, superoxide dismutase (150 U/ml, 6 h) effected a similar

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Fig. 3. Effect of simvastatin on LPS-induced EC permeability and cytoskeletal rearrangement. 
A: compared with 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, 16 h) and Rho kinase inhibition (Y-27632, 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 geranylgeranyl pyrophosphate (GGPP; 10 μM, 16 h). 
B: EC monolayer protection by simvastatin corresponded to early evidence of decreased LPS-induced (1 μg/ml, 1.5 h) paracellular gaps by immunofluorescence (white arrows) in simvastatin-treated EC (5 μM, 16 h).
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 p47phox translocation to the cell membrane, suggesting that these events are necessary but not sufficient for membrane NADPH oxidase activity. Moreover, whereas simvastatin treatment resulted in both Rac1 and p47phox 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 NOx-based oxidase, which is constitutively active and does not rely on p47phox or other regulatory subunits for activation (30).

Finally, we sought to determine whether 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 (Fig. 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 (Fig. 5A). This effect was even more pronounced than that observed with diphenyleneiodonium, 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) (Fig. 5B). Collectively these results suggest that these findings are attributable to the inhibition of geranylgeranylation by simvastatin rather than any potential nonspecific effects. Importantly, simvastatin did not affect a significant change in total Rac as measured by the combined densitometry of membrane and cell

Fig. 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 with control cells, superoxide production in LPS-treated (100 ng/ml, 6 h) EC is associated with a marked increase in superoxide production as measured by dihydroethidium (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 with controls as measured by FITC-dextran monolayer permeability (*P < 0.05). (n = 3 for each condition).
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 (100 ng/ml, 6 h) superoxide generation by simvastatin (5 μM, 16 h) is comparable to effects of small interfering RNA (siRNA) specific for Rac1 (*P < 0.05 and **P < 0.05). (n = 3 for each experimental condition). ns RNA, nonspecific siRNA.

**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 nonspecific siRNA as controls, siRac1 effected an ~20% decrease in thrombin-induced (1 U/ml, 1 h) EC permeability as measured by FITC-dextran Transwell permeability (Fig. 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 before 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 with 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 (Fig. 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). Our laboratory previously reported novel lung vascular protection conferred by simvastatin in a murine model of ALI (22), results subsequently validated by other reports (11, 44). How-
ever, 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). Although 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, 40). 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 (21). 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, our laboratory was previously unable to detect evidence of EC barrier protection by simvastatin with pretreatment durations of <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 mem-

![Graph](Image)
brane 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 present studies, thrombin and LPS. Although 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 nonspecific effects. Finally, our conclusion that the protective effects of simvastatin in vivo are not solely due to Rac1 inhibition and that 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 affects 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 peroxynitrite (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, our laboratory has previously confirmed that the attenuation of agonist-induced EC permeability by simvastatin is unaffected by 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 present studies, thrombin and LPS. Although 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 nonspecific effects. Finally, our conclusion that the protective effects of simvastatin in vivo are not solely due to Rac1 inhibition and that 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).

The limitations of our present study include the reliance on the use pharmacological inhibitors in several experiments from which our conclusions are drawn. We recognize the potential nonspecific 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. Although our data suggest 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 also RhoA-GTP as well (6, 9). Ultimately, because 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.

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