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Simvastatin inhibits *Staphylococcus aureus* host cell invasion through modulation of isoprenoid intermediates

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Abstract

Patients on a statin regimen are at a decreased risk of death due to bacterial sepsis. We have found that protection by simvastatin includes the inhibition of host cell invasion by *Staphylococcus aureus*, the most common etiologic agent of sepsis. Inhibition was due in part to depletion of isoprenoid intermediates within the cholesterol biosynthesis pathway and led to the cytosolic accumulation of the small-guanosine triphosphatases (GTPases) CDC42, Rac, and RhoB. Actin stress fiber disassembly required for host invasion was attenuated by simvastatin and by the inhibition of phosphoinositide 3-kinase (PI3K) activity. PI3K relies on coupling to prenylated proteins, such as this subset of small-GTPases, for access to membrane-bound phosphoinositide to mediate stress fiber disassembly. Therefore, we examined whether simvastatin restricts PI3K cellular localization. In response to simvastatin, the PI3K isoform p85, coupled to these small-GTPases, was sequestered within the cytosol. From these findings, we propose a mechanism whereby simvastatin restricts p85 localization, inhibiting actin dynamics required for bacterial endocytosis. This may provide the basis for protection at the level of the host in invasive infections by *S. aureus*.

Introduction

Death due to sepsis is as common as death due to myocardial infarction in the US each year (Angus et al., 2001). The most prevalent etiologic agent, *S. aureus* (Diekema et al., 2001), is a leading hospital-acquired pathogen with community-acquired infection becoming increasingly widespread (Lowy, 1998). The incidence of complicated infection has increased with the development of invasive procedures and with increased aging, immunocompromised patient populations. Bacterial sepsis is complicated further by invasive endocarditis and osteomyelitis, with persistent infections commonly requiring lengthy hospital stays and treatment. The emergence of antibiotic-resistant strains, including methicillin resistant *S. aureus* (MRSA), has limited treatment options. Unexpectedly, statins, a class of drugs generally prescribed for lowering cholesterol, may provide a new adjunctive therapeutic for bacterial sepsis (Liappis et al., 2001; Kruger and Merx, 2007).

The pharmacology of statins includes effects due to the lowering of plasma cholesterol as well as to the depletion of intermediates within the cholesterol biosynthesis pathway. Statins lower cholesterol through inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in cholesterol biosynthesis (Tobert, 2003). In addition to the reduction in cholesterol levels, inhibition diminishes the synthesis of intermediates within the pathway, including mevalonate and the hydrophobic isoprenoids geranylgeranyl pyrophosphate (GGpp) and farnesyl pyrophosphate (Fpp) (Goldstein and Brown, 1990). Pleiotropic benefits independent of cholesterol lowering have been associated with this depletion of isoprenoid intermediates. Isoprenoids function as membrane anchors and in protein-protein interactions. During post-translational prenylation, 10 carbon GGpp or 15 carbon Fpp is covalently added at the cysteine residue of the conserved carboxyl terminal motif CaaX, where “C” designates the prenylated cysteine residue, “a” designates aliphatic residues, and “X” determines whether the target is recognized by geranylgeranyl or farnesyltransferases (Zhang and Casey, 1996). With statin treatment, the pool of GGpp and Fpp is reduced, diminishing prenylation, sequestering CaaX-containing proteins within the cytosol and thereby impairing functions that require membrane localization.

At the cell membrane, the interaction between the CaaX-containing proteins Rac and CDC42 and the PI3K isoform p85 (Zheng et al., 1994; Bokoch et al., 1996) may facilitate actin dynamics and endocytic trafficking. p85 is a regulatory subunit within the PI3K family of proteins (Vanhaesebroeck and Waterfield, 1999). The regulatory subunits, which include p85 α , and β ; p55 α and γ ; and p50 α , possess domains for interaction with membrane-bound proteins and for heterodimerization with catalytic subunits, p110 α , β and δ . Regulatory subunits provide a linkage between membrane-bound proteins and the catalytic subunits. These catalytic subunits phosphorylate membrane-bound phosphoinositides, including phosphoinositide (PI) 4,5-bisphosphate, forming PI 3,4,5-trisphosphate (PIP₃). By coupling through the Rho-GAP domain of p85, a domain missing in the truncated p55 and p50 isoforms, prenylated small-GTPases would be well-positioned to bring the catalytic p110 domains in proximity with membrane-bound phosphoinositides. PI3K may function in this manner as an intermediary to regulate actin dynamics and initiate endocytosis (Johnson, 1999) as membrane-bound PIP₃ binds to α -actinin, dislodging this bundling protein from actin filaments and disassembling actin stress fibers (Fraley et al., 2005). Thus, prenylation of a subset of CaaX-containing proteins coupled to p85 possibly localizes PI3K at the cell membrane to facilitate the reordering of actin stress fibers.

Actin dynamics can drive both clathrin and non-clathrin mediated endocytosis, cellular processes exploited by pathogenic bacteria for host invasion (Sinha and Herrmann, 2005; Nitsche-Schmitz et al., 2007). To initiate invasion, surface adhesins of pathogenic *S. aureus* bind to extracellular matrix proteins. As matrix-bound *S. aureus* engages host receptors, the pathogen enters the cell during endocytosis of matrix/receptor complexes. Although *S. aureus* primarily has been considered an extracellular pathogen, it is increasingly clear that invasiveness contributes to pathogenesis (Lowy, 1998; Alexander and Hudson, 2001; Foster, 2005; Que et al., 2005; Sinha and Herrmann, 2005; Hauck and Ohlsen, 2006; Proctor et al., 2006) and blocking receptor engagement is an emerging immunotherapeutic target (Rivas et al., 2004).

We explored whether statins inhibit endocytic invasion by *S. aureus*. Our hypothesis is that by diminishing protein prenylation, simvastatin sequesters CaaX-containing proteins coupled to PI3K within the cytosol, inhibiting actin dynamics required for *S. aureus* endocytic invasion. We have examined whether pretreatment of host cells with simvastatin at therapeutic concentrations prevents *S. aureus* invasion and the mechanism of this inhibition.

Methods

Chemicals

The following were used at the concentrations and durations indicated within each figure or method described below. Simvastatin, geranylgeranyl transferase inhibitor GGTI-2147 and farnesyl transferase inhibitor FTI-277 (Calbiochem, San Diego, CA); dimethyl sulfoxide and NaCl (Fisher Scientific, Pittsburgh, PA); tryptic soy agar, saponin, cholesterol, mevalonate, geranylgeraniol, farnesyl pyrophosphate, lysostaphin, gentamicin, PIPES, EGTA, KCl, Triton X-100, Tween-20, bovine serum albumin, and LiCl (Sigma, St. Louis, MO); LY294002 (Cell Signaling, Danvers, MA); Mini-Tab (Roche, Indianapolis, IN); MgCl₂ (VWR, West Chester, PA), phosphate buffered saline (PBS), Tris-HCl (Invitrogen, Carlsbad, CA); secramine A (Xu et al., 2006).

Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC, Cascade Biologics, Portland, OR) were grown in M200 medium supplemented with Low Serum Growth Supplement (LSGS, Cascade Biologics, 5% CO₂, 37°C, 75 cm² vented cap flasks) prior to plating. For invasion assays and flow cytometry, 10⁵ cells were plated on 35 mm tissue culture dishes (Fisher) coated with Attachment Factor (a gelatin that contains no extracellular matrix proteins, Cascade Biologics). For confocal imaging, cells were plated as above on 35 mm glass-bottom dishes (MatTek, Ashland, MA) coated as above. Simvastatin treatments were initiated on day 3 of plating. LY294002 and secramine A treatments were performed on day 4.

Invasion assay

S. aureus (29213, American Type Culture Collection, Manassas, VA) were subcultured daily in tryptic soy broth (200 rpm, 37°C). Bacteria were harvested by centrifugation (10000 rpm, 3 min, 37°C), washed once, and resuspended to 3×10⁸ cells/ml in saline. HUVEC were washed once with 1× PBS and incubated with *S. aureus* (1.2×10⁸) in 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA)/PBS (1 h, 5% CO₂, 37°C). Invasion was terminated by incubation with lysostaphin (20 µg/ml) and gentamicin (50 µg/ml) in 10% FBS/PBS (45 min, 5% CO₂, 37°C). Intracellular bacteria were released into the medium using 1% saponin/PBS (20 min, 5% CO₂, 37°C). Serial dilutions of the medium were plated on tryptic soy agar and colony counts performed (16 h, 37°C). All invasion assays were performed on day 4 of plating. To assess whether compounds were bactericidal, *S. aureus* (1.2 × 10⁸ cfu) were incubated with compounds at the concentrations indicated in 10% FBS (1 h), and serial dilutions plated on tryptic soy agar.

Generation of CDC42 C507V/V5

Site-directed mutagenesis (QuickChange, Agilent Technologies, Santa Clara, CA) of human CDC42 cDNA in pUSE (Upstate, Lake Placid, NY) was performed to substitute the cysteine required for prenylation at position 507 with a valine, remove the stop codon, and fuse to the V5 tag (Invitrogen). The following primer sequence was used: ccg aag aag agc cgc agg gta gtg ctg cta cta ggt aag cct atc cct aac cct ctc ctc ggt cta gat tct acg tga ctc gag tct aga ggg ccc g. Human embryonic kidney (HEK) 293A cells (Invitrogen) were transfected with CDC42 C507V/V5/pUSE using Lipofectamine (Invitrogen) and a stable cell line created from a geneticin-resistant clone. Invasion assays were performed as described above with the exception that following the gentamicin/lysostaphin treatment, cells were suspended using trypsin and pelleted prior to the saponin addition.

Cytotoxicity assay

HUVEC or HEK 293A were pretreated with compounds at the concentrations indicated in figure legends and cytotoxicity assessed using flow cytometry. Propidium iodide uptake was measured in 1×10^4 cells/treatment.

Immunofluorescence

Following infection, HUVEC were washed with 1x PBS, fixed (4% paraformaldehyde/PBS, 30 min, Electron Microscopy Sciences, Hatfield, PA), permeabilized, blocked (0.1% Triton, 1% bovine serum albumin, 30 min), and incubated with Alexa Fluor 488 phalloidin for actin (1:40, Invitrogen). Confocal images were acquired using an inverted Zeiss Axiovert200 microscope equipped with a plan-apochromat 40 $\times$, 1.2 NA water immersion lens with correction collar and LSM 5 Pascal scan head. Alexa 488 was excited by the 488nm Ar laser line and detected using a 505–530nm bandpass filter. Z-sectioning and frame size were set to Nyquist sampling. Maximum pixel projections from the Z-stacks were generated and analyzed for actin morphology. Differential interference contrast images were acquired simultaneously with fluorescence images.

Adenoviral constructs

Wild-type human PI3Kp110 α in pDONR201 was kindly provided by Dr. Jim Thomas at Eli Lilly & Co. By site-directed mutagenesis (QuickChange), the catalytic domain was truncated at amino acid 915 to remove the region that putatively interacts with the phosphoinositide polar head group for lipid kinase activity (Yart et al., 2002). A point mutation was generated to substitute lysine with arginine at amino acid 805, the site required for protein kinase activity (Bondeva et al., 1998). Mutated p110 α was cloned into pAD/CMV/V5 through LR recombination (Gateway System, Invitrogen). Kinase-dead p110 α /pAD/CMV/V5 and LacZ/pAD/CMV/V5 (Invitrogen) were transfected into HEK 293A using Lipofectamine, viral particles harvested, purified (BD Biosciences, Franklin Lakes, NJ), and titer determined. Optimal multiplicity of infection (MOI) and duration of infection were determined for both constructs by infecting HEK293A and assessing expression by western blot analysis. Invasion assays were performed as described above for assessing CDC42 C507V/V5.

Cell fractionation

HEK 293A cells were plated in DMEM/10%FBS at a density of 7.5×10^5 cells on 100 mm tissue culture dishes (Fisher) coated with Attachment Factor. At day 3, cells were pretreated with DMSO or simvastatin (1 μ M). On day 4, cells were washed once with ice cold 1 $\times$ PBS, incubated on ice in buffer (15 min; 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 1.25 mM EGTA, and 10 mM PIPES, pH 7.3; 1 x Mini-Tab) and harvested using cell scrapers. Following sonication (10 sec, 40TL probe, Ultrasonic Power Corporation, Freeport, Illinois), lysates were pelleted (500 $\times$ g, 5 min, 4°C) and supernatants centrifuged (1 h, 110,000 $\times$ g, 4°C, Beckman SW41). The supernatant was designated as the cytosolic fraction. Total protein concentration was determined using the BIO-RAD Protein Assay (Hercules, CA).

Western blot analysis

Cytosolic and membrane fractions were subjected to electrophoresis using NU-PAGE (1 $\times$ MES buffer), and transferred to PVDF (Invitrogen). Membranes were blocked using Odyssey Blocking Buffer (LI-COR, Lincoln, NE, RT, 1 h) then incubated in Blocking Buffer/0.1% Tween-20 (4°C, 16 h) with anti-CDC42 (#610928, BD Biosciences), with anti-Rac (#05–389, Upstate/Millipore, Temecula, CA), or anti-RhoB (sc-180, Santa Cruz, Santa Cruz, CA), and subsequently with anti-flotillin-1 (#610820, BD) and anti-actin (Chemicon/Millipore). Membranes were washed in 1 $\times$ PBS/0.1% Tween-20 and incubated with anti-mouse IRDye 800CW (Rockland Immunochemicals, Gilbertsville, PA) for CDC42, Rac, and actin; with anti-rabbit IRDye 800CW (Rockland Immunochemicals) for RhoB; and with anti-mouse Alexa Fluor 680 for flotillin and/or actin (1:40000, RT, 1 h, in Blocking Buffer/0.1% Tween-20). Membranes were washed in 1 $\times$ PBS/0.1% Tween-20, and fluorescent signals (680 and 800) detected using the Odyssey Infrared Imaging System (LI-COR). Integrated intensity values were acquired using Odyssey software (LI-COR).

Immunoprecipitation

To assess coupling between PI3K p85 and CDC42, Rac, or RhoB, cytosolic fractions (500–1000 μ g), were incubated overnight with anti-CDC42, anti-Rac, or anti-RhoB, and immunocomplexes recovered using Protein A Agarose (Invitrogen). Following extensive washing with ice cold 1 $\times$ PBS and subsequently with ice cold LiCl buffer (500 mM LiCl/100 mM Tris-HCl, pH 7.5), immunocomplexes were retrieved by boiling in LDS-Sample Buffer containing reducing agent (Invitrogen). Immunocomplexes were assessed by western blot analysis as described above, probed with anti-PI3K p85 (#06–497, Upstate/Millipore, 16 h, 4°C) followed by anti-rabbit IRDye 800CW (1:40000, RT, 1 h) and detected using the Odyssey Infrared Imaging System. Integrated intensity values were acquired for p85 bands and background using Odyssey software.

Statistical analyses

Normally distributed data from invasion assays were analyzed using one-way ANOVA followed by Student-Neuman-Keuls post-hoc analysis (Sigma Stat, Systat, Point Richmond, CA). Actin stress fibers were evaluated in 200 cells/treatment from randomly selected fields using an inverted fluorescent microscope and data assessed using χ^2 test of association

(SPSS, Inc., Chicago, IL). Differences between groups were considered statistically significant at $p = 0.05$.

Results

Simvastatin prevents *S. aureus* host cell invasion through inhibition of isoprenoid production

Nanomolar amounts of simvastatin inhibited *S. aureus* invasion (Fig. 1). The doses used were neither cytotoxic to HUVEC nor bactericidal (Fig. 1, Panels B and C). We examined whether the inhibition by simvastatin was due to diminished availability of intermediates within the cholesterol biosynthesis pathway or to diminished levels of cholesterol. Replenishing mevalonate, the product of HMG-CoA reduction, restored *S. aureus* invasiveness (Fig. 2, Panel A). In contrast, replenishing cholesterol failed to restore *S. aureus* invasion (Fig. 2, Panel B). Host cell invasion was restored with replenishment of the dephosphorylated form of GGpp, GGOH, and partially restored with Fpp (Fig. 2, Panels C and D). Inhibition of geranylgeranylation and farnesylation decreased invasiveness (Fig. 2, Panels E and F).

Inhibiting the membrane insertion of CDC42 decreases host cell invasion

CDC42 is a CaaX-containing protein known to be activated early during *S. aureus* invasion (Arbibe et al., 2000) and to mediate actin dynamics (Johnson, 1999). To determine whether membrane-localization by prenylated CDC42 is required for *S. aureus* invasion, HEK 293A cells were pretreated with secramine A (1 h), an antagonist of prenylated CDC42 membrane association (Xu et al., 2006). Secramine A inhibited *S. aureus* invasion (Fig. 3, Panel A) at concentrations that were neither cytotoxic (Fig. 3, Panel B) nor bactericidal (data not shown). To assess directly the role of prenylated CDC42 in *S. aureus* invasion, a mutated construct was generated in which the cysteine residue required for prenylation was substituted with a valine and expressed as a stable clone. Expression of CDC42 C507V/V5 inhibited *S. aureus* invasion (Fig. 3, Panel C).

Pretreatment with simvastatin (20 h) inhibited actin stress fiber disassembly during *S. aureus* invasion

At day 4 of plating, HUVEC displayed extensive stress fiber formations (Fig. 4, Panel A). During invasion, stress fibers disassembled (Fig. 4, Panel A), and disassembly persisted throughout 2 h of invasion (Fig. 4, Panel B). In contrast, stress fibers remained intact in control cells over time (Fig. 4, Panel B). Stress fibers depolymerized in the presence of heat killed *S. aureus* (data not shown). Simvastatin reduced actin stress fiber disassembly (Fig. 4, Panel C).

Inhibition of PI3K decreases actin stress fiber disassembly and *S. aureus* invasiveness

The inhibition of PI3K lipid-kinase activity using LY294002 restricted the disassembly of actin stress fibers during invasion (Fig. 5, Panel A). In a dose-dependent manner, LY294002 inhibited *S. aureus* host cell invasion (Fig. 5, Panel B). Adenoviral expression of a kinase-dead form of the PI3K isoform p110 α inhibited *S. aureus* invasion by 70%, more than the 45% decrease in response to adenoviral expression of the control LacZ (Fig. 5, Panel C).

Simvastatin increases the accumulation of small-GTPases coupled to PI3K within the cytosol

Cell fractionation was performed in order to assess the localization of CDC42. In the absence of simvastatin, cytosolic CDC42 is minimal (Fig. 6, Panel A). In response to simvastatin pretreatment, CDC42 accumulated in the cytosol (Fig. 6, Panel A). Constitutively, Rac is present at the membrane and within the cytosol. In response to simvastatin, Rac localized primarily within the cytosol (Fig. 6, Panel B). RhoB is primarily membrane-associated in the absence of simvastatin, accumulating in the cytosol following pretreatment (Fig. 6, Panel B). The membrane marker, flotillin, was detected only in the membrane samples (Fig. 6, Panel B). Actin was detected in both cytosolic and membrane fractions, diminishing in the membrane fraction and accumulating in the cytosol in response to simvastatin (Fig. 6, Panels A and B). Because CDC42 activation is immediate and transient in response to *S. aureus* (Arbibe et al., 2000), membrane and cytosolic fractions from cells pretreated with simvastatin or DMSO were assessed upon initial infection (2 min). In the presence of simvastatin, CDC42, Rac, and Rho B, remained sequestered in the cytosol with infection (Fig. 6, Panel B).

To evaluate whether the cytosolic accumulation of these small-GTPases led to sequestration of PI3K p85 within the cytosol, each small-GTPase was immunoprecipitated from the cytosolic fraction, immunocomplexes subjected to PAGE, and blots probed for associated p85. In response to simvastatin, p85 coupled to CDC42, Rac, and RhoB, was more abundant within the cytosolic fraction (Fig. 6, Panel C).

Discussion

In this study, we have addressed the question of whether the pharmacology of statins extends to the inhibition of host cell invasion by *S. aureus*. Our findings indicate that at nanomolar concentrations within the therapeutic range, simvastatin inhibits invasion by a mechanism that is neither cytotoxic nor bactericidal (Fig. 1). Inhibition is dependent upon mevalonate, an early product within the cholesterol biosynthesis pathway and is due in part to depletion of the isoprenoid intermediates GGpp and Fpp as replenishment of each restored invasion and inhibition of geranylgeranyl or farnesyl transferase decreased invasiveness (Fig. 2). Secramine A, which blocks membrane-association by prenylated CDC42, inhibited invasion, as did expression of CDC42 C507V/V5, a mutated construct deficient in the site for prenylation (Fig. 3). Taken together, these findings support the concept that inhibition of the prenylation-dependent localization of this CaaX-containing protein is central to inhibition of invasion. The mechanism of inhibition by simvastatin included the attenuation of actin stress fiber disassembly required for endocytosis of the bacterium (Fig. 4). As PI3K mediates stress fiber disassembly and invasion (Fig. 5), we examined whether simvastatin was affecting PI3K cellular localization. PI3K lacks prenylation domains but can rely on coupling to prenylated proteins, such as a subset of small-GTPases, for access to membrane-bound phosphoinositide. Simvastatin led to the cytosolic accumulation of the small-GTPases CDC42, Rac, and RhoB, coupled to the PI3K isoform p85 (Fig. 6). From these findings, we propose a mechanism whereby simvastatin restricts p85 localization, inhibiting actin dynamics required for caveolar endocytosis. This

may provide the basis for protection at the level of the host in invasive infections such as endocarditis and osteomyelitis as well as in the treatment of bacterial sepsis.

Complex actin dynamics mediate the endocytosis of *S. aureus* (Alexander and Hudson, 2001; Schroder et al., 2006) possibly generating the pulling forces necessary for inward movement and vesicular trafficking (Agerer et al., 2005). Inhibition of actin stress fiber depolymerization by LY294002 (Fig. 5, Panel A) indicated that these actin dynamics are in part regulated via a PI3K p110 catalytic domain, as the inhibitory action of this compound is within the ATP-binding site of the p110 domain (Walker et al., 2000). Sequestering the regulatory subunit p85 within the cytosol could impair p110 catalytic activity by restricting access to membrane-bound phosphoinositide. p85 also could affect actin dynamics independently of the p110 subunits as p85 is functional as an unbound monomer (Brachmann et al., 2005). Our data indicate a continuous loss of fibers during the invasion by *S. aureus* (Fig. 4, Panel B) which corresponds temporally with the recent report that bridging between *S. aureus* and the integrin receptor $\alpha 5 \beta 1$ results in sustained actin dynamics (Schroder et al., 2006). Bacteria were found to spend on average 45 min on the endothelial cell surface with stimulation of actin waves, formation of actin cups and generation of comet tails prior to endocytosis of the bacterium. The current findings indicate that depletion of isoprenoid intermediates by simvastatin and inhibition of PI3K catalytic activity attenuates the cortical actin dynamics required for invasion by the bacterium.

An early event in *S. aureus* invasion is the activation of CDC42 (Arbibe et al., 2000), a CaaX-containing small-GTPase known to facilitate actin dynamics (Johnson, 1999). CDC42 stimulates actin dynamics through a number of effectors. One potential effector is PI3K. CDC42 couples to PI3K through the Rho-GAP domain of PI3Kp85 and it has been postulated that the interaction determines appropriate cellular localization for the lipid kinase (Zheng et al., 1994). Therefore, we examined the possibility that simvastatin inhibits *S. aureus* invasion by impeding the access of PI3K to membrane-bound phosphoinositide. Nanomolar concentrations of simvastatin were sufficient to significantly inhibit invasion, suggesting that the depletion of isoprenoid intermediates impairs the translocation of multiple prenylated proteins, possibly coupled to distinct PI3K isoforms. In addition to CDC42, Rac had previously been found to couple with PI3K within the Rho-GAP domain of p85 (Bokoch et al., 1996), and, more recently, both Rac and RhoB were found to localize within the cytosol in response to simvastatin (Stamatakis et al., 2002; Cordle et al., 2005). Interaction between p85 and Rac or CDC42 requires GTP loading of these small-GTPases. Interestingly, Cordle et al., found that simvastatin stimulates GTP loading of Rac, while the small-GTPase remained functionally inactivated and translocation to the cell membrane inhibited. *S. aureus* likewise stimulates an immediate and transient GTP loading of CDC42 (Arbibe et al., 2000). Therefore, in response to either simvastatin or *S. aureus*, the requirement for GTP loading of small-GTPases for interaction with p85 appears to be met and our findings indicate that this association sequesters p85 within the cytosol in response to simvastatin.

In addition to the small-GTPases examined in the current study, additional candidates that may be mediating inhibition include the γ -subunit of heterotrimeric G-proteins that couple to the PI3K isoforms p110 γ and β and which require prenylation for membrane insertion

(Zhang and Casey, 1996) and small-GTPases capable of coupling with p85 through the Rho-GAP domain that have yet to be identified. Our findings also reveal a role for the p110 catalytic domain as treatment with LY294002 or adenoviral expression of a kinase-dead form of p110 α decreased invasion more than DMSO or LacZ controls respectively (Fig. 5, Panel B). Adenoviral expression of LacZ diminished infection relative to DMSO control by 45% (Fig. 5, Panel B), supporting the concept that the endocytic pathway utilized for adenoviral uptake is shared by *S. aureus* for invasion (Sinha and Herrmann, 2005). Previous work has clearly demonstrated that the acute effect of simvastatin on the PI3K signaling pathway is the activation of the downstream mediator Akt (Kureishi et al., 2000). Our study reveals that the pharmacology of long-term (20 h) statin treatment includes sequestration of PI3K through coupling with CaaX-containing proteins.

Although a number of clinical studies indicate an improved prognosis for individuals on a statin regimen, the safety and efficacy of statin therapy during sepsis has been questioned (Mahboobi et al., 2006; Vincent and Miller, 2006; Bromilow and Schuster Bruce, 2007). Concerns include the altered pharmacokinetics of statins in critically ill patients that result in high levels of circulating, non-metabolized drug, predisposing this patient population to myopathy and rhabdomyolysis. Large, clinical studies will begin to address these concerns. In addition, developing an understanding of the underlying mechanism of statin pharmacology will aid in determining whether statins are an efficacious adjunctive therapy in infectious disease and may provide the rationale for the development of more targeted therapeutics.

Invasion by *S. aureus* facilitates persistent, relapsing infection (Clement et al., 2005; Proctor et al., 2006) and in the case of endocarditis, is central to pathogenesis (Yeaman and Bayer, 2000; Que et al., 2005). Immunotherapeutics directed against bacterial adhesin molecules have shown clinical promise, and it is postulated that the protective effect is due in part to impaired endothelial invasion (Rivas et al., 2004). The current study raises the possibility that inhibition of invasion by simvastatin could improve extracellular antibiotic efficacy, reduce hematogenous spread, and impede intracellular pathogenicity including persistent infection. Previous work had shown that statin treatment during sepsis also improves cardiovascular function and modulates the immune response (Kwak et al., 2000; Merx et al., 2004; Jacobson et al., 2005). Taken together, these findings reveal that the pharmacology of simvastatin extends to the inhibition of host invasion and provide a basis for further studies into the usefulness and efficacy of simvastatin, as well as therapeutics targeting distinct PI3K isoforms, as adjunctive approaches in the treatment of invasive infection.

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Abbreviations

GTPases	small-guanosine triphosphatases
PI3K	phosphoinositide 3-kinase
PI	phosphoinositide
PIP₃	PI 3,4,5-trisphosphate
MRSA	methicillin resistant <i>S. aureus</i>
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
GGpp	geranylgeranyl pyrophosphate
Fpp	farnesyl pyrophosphate

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Figure 1. Simvastatin inhibits *Staphylococcus aureus* host cell invasion

Panel A. Human umbilical vein endothelial cells (HUVEC) were pretreated with simvastatin (0.1 or 1.0 μ M, 20 h) or vehicle control (dimethyl sulfoxide, DMSO, 20 h) followed by *S. aureus* infection (1 h). Extracellular bacteria were removed by the bactericides gentamicin and lysostaphin. Intracellular bacteria were released from HUVEC into the medium by the detergent saponin and serial dilutions of the medium were plated on tryptic soy agar for colony counts (16 h). Data are % control $\pm$ SEM and are representative of three separate experiments (* p < 0.05 compared to control, n = 3/treatment by one-way

ANOVA and Student-Neuman-Keuls post-hoc analysis). **Panel B.** *Simvastatin treatment is non-cytotoxic.* HUVEC were pretreated with simvastatin (0.1 or 1.0 μM) or DMSO (20 h) and cytotoxicity assessed using flow cytometry to detect the uptake of propidium iodide. 1×10^4 cells were counted per treatment. **Panel C.** *Simvastatin treatment is non-bactericidal.* *S. aureus* (1.2×10^8 cfu) were treated with simvastatin (0.1, 1.0, or 10 μM) or DMSO (1 h), and serial dilutions plated on tryptic soy agar. Data are colony counts $\pm$ SEM.

Figure 2. Decreased isoprenoid production inhibits *Staphylococcus aureus* host cell invasion

Panel A. Mevalonate reverses simvastatin inhibition of *S. aureus* host cell invasion. Human umbilical vein endothelial cells (HUVEC) were pretreated (20 h) with the vehicle control dimethyl sulfoxide (DMSO), simvastatin (0.5 μ M), or simvastatin supplemented with mevalonate (100 μ M), the product of HMG-CoA reduction. Cells were incubated with *S. aureus* for 1 h. Following infection, extracellular bacteria were removed by the bactericides gentamicin and lysostaphin. Intracellular bacteria were released from HUVEC into the medium by the detergent saponin and serial dilutions of the medium were plated on tryptic

soy agar for colony counts. **Panel B.** *Cholesterol fails to reverse simvastatin inhibition of S. aureus invasion.* HUVEC were pretreated (20 h) with DMSO, simvastatin (0.5 μ M) alone or in the presence of cholesterol (100 μ M). Cells were infected with *S. aureus* (1 h), intracellular bacteria recovered and colony counts performed as described above. **Panel C.** *Geranylgeraniol (GGOH) restores host cell invasion.* HUVEC were pretreated with simvastatin alone (0.1 μ M, 20 h) or in the presence of GGOH, an isoprenoid intermediate (5 μ M, last 6 h). Cells were infected (1 h) and invasiveness assessed as described above. **Panel D.** *Farnesyl pyrophosphate (Fpp) partially restores invasiveness.* HUVEC were pretreated with simvastatin alone (0.1 μ M, 20 h) or in the presence of the isoprenoid intermediate Fpp (5 μ M, last 6 h). Cells were infected (1 h) and invasiveness assessed as described above. **Panels E and F.** *Inhibition of geranylgeranyl transferase or farnesyl transferase decreases S. aureus host cell invasion.* HUVEC were pretreated (6 h) with geranylgeranyl (GTI, 10 μ M) or farnesyl transferase inhibitors (FTI; 5 μ M) or DMSO, infected (1 h) and colony counts performed as above. Data are representative of replicate experiments and are presented as percent control. One-way ANOVA and Student-Neuman-Keuls post-hoc analysis were performed, n = 3–5/treatment (*different than DMSO control; †less than GGOH/Fpp in the presence of simvastatin; p < 0.05).

Figure 3. Antagonism of prenylated CDC42 membrane association inhibits host cell invasion

Panel A. HEK 293A were pretreated with the vehicle control dimethyl sulfoxide (1 % DMSO) or secramine A (10 μ M, 1 h), a compound that inhibits membrane association by prenylated CDC42 (Xu et al., 2006). Cells were incubated with *S. aureus* for 1 h, extracellular bacteria removed by the bactericides gentamicin and lysostaphin, and intracellular bacteria released from the host cells into the medium by the detergent saponin. Serial dilutions of the medium were plated on tryptic soy agar for colony counts. **Panel B.** *Inhibitory concentrations of secramine A are non-cytotoxic.* HEK 293A were pretreated

with 1% DMSO or secramine A (3, 10, or 30 μ M, 1 h) and cytotoxicity assessed by propidium iodide uptake in 1×10^4 cells per treatment. **Panel C.** *Expression of CDC42 C507V/V5, a mutated construct lacking the prenylation site, inhibits invasion.* Stably transfected HEK 293A expressing CDC42 C507V/V5 were incubated with *S. aureus* (1 h) and invasion assessed as in Panel A. Data are representative of replicate experiments. One-way ANOVA followed by Student-Neuman-Keuls post-hoc analysis were performed, $n = 3-5$ /condition (*less than control, $p < 0.05$).

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Figure 4. Simvastatin limits disruption of actin stress fibers during *Staphylococcus aureus* host cell invasion

Panel A. *Actin stress fibers disassemble during infection.* Human umbilical vein endothelial cells (HUVEC) were infected with *S. aureus* (1 h). Following invasion, extracellular bacteria were removed using the bactericides lysostaphin and gentamicin. HUVEC were fixed, permeabilized, and blocked. Actin was detected using Alexa Fluor 488 phalloidin. Scale bar is 10 μ M. **Panel B.** *Stress fiber disassembly continues throughout infection.* HUVEC were infected with *S. aureus* for times indicated. Actin stress fibers were evaluated in 200 cells in

uninfected or infected samples from randomly selected fields. **Panel C.** *Simvastatin attenuates actin stress fiber disassembly.* HUVEC were pretreated (20 h) with simvastatin (1.0 μ M) or vehicle control (dimethyl sulfoxide, DMSO), infected with *S. aureus* (1 h), and actin stress fibers evaluated as in Panel B. Data are presented as the % of cells displaying actin stress fiber disassembly (*different than control; $p < 0.05$ by χ^2 test of association).

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Figure 5. Inhibition of phosphoinositide 3-kinase (PI3K) attenuates actin stress fiber disassembly and *Staphylococcus aureus* host cell invasion

Panel A. Human umbilical vein endothelial cells (HUVEC) were pretreated (1 h) with vehicle control (dimethyl sulfoxide, DMSO) or with LY294002 (50 μ M), infected with *S. aureus* (1 h), and extracellular bacteria removed using gentamicin and lysostaphin. HUVEC were fixed, permeabilized, and actin detected using phalloidin 488. Stress fibers were evaluated in 200 cells from randomly selected fields. Data are presented as the % of cells displaying actin stress fiber disassembly (*less than infected control; $p < 0.05$ by χ^2 test of

association). **Panel B.** *LY294002 decreases host cell invasion in a dose dependent manner.* HUVEC were pretreated with DMSO or LY294002 (10, 25, or 50 μ M, 1 h), and infected with *S. aureus* (1 h). Following infection, extracellular bacteria were removed by the bactericides gentamicin and lysostaphin. Intracellular bacteria were released from HUVEC into the medium by the detergent saponin, serial dilutions of the medium were plated on tryptic soy agar and colonies counted. **Panel C.** *Adenoviral expression of kinase-dead PI3Kp110 α inhibits invasion.* HEK293 were pretreated with DMSO or LY294002 (50 μ M, 1 h), or exposed to LacZ or kinase-dead p110 α (KDp110 α) adenovirus (24 h), infected with *S. aureus* (1 h), and invasion assessed as in Panel B. Inset is immunoblot of lysate from HEK 293A following exposure to KDp110 α adenovirus (24 h), probed with anti-p110 α , showing endogenous p110 α compared to truncated mutant expression. Data are representative of replicate experiments, n = 3/treatment (* less than infected DMSO control; †less than LacZ control; $p < 0.05$ by one-way ANOVA with Student-Neuman-Keuls post-hoc analysis).

Figure 6. Simvastatin leads to the accumulation of CDC42, Rac, and RhoB, coupled to PI3K p85 within the cytosol

Panel A. HEK 293A were pretreated (20 h) with the vehicle control dimethyl sulfoxide (DMSO) or with simvastatin (1.0 μM), cytosolic fractions isolated and western blot analysis performed loading 15 μg total protein/sample. The immunoblot was probed with anti-CDC42 and anti-mouse IRDye 800CW, followed by anti-actin and anti-mouse Alexa Fluor 680. **Panel B.** Membrane and cytosolic fractions from cells pretreated with simvastatin (10 μM) or DMSO in the absence or presence of infection (2 min) were subjected to SDS-PAGE

and probed with antibodies to Rac, RhoB, actin, and the membrane marker flotillin, followed by fluorophore-conjugated secondary antibodies. **Panel C.** Cytosolic lysates were immunoprecipitated (IP) using anti-CDC42, anti-Rac, or anti-RhoB, and immunocomplexes subjected to SDS-PAGE. Immunoblots (IB) were probed with anti-PI3K p85 followed by Alexa Fluor 680 goat anti-rabbit. Fluorescence was detected using the Odyssey Infrared Imaging System. Integrated intensities and background correction were performed using Odyssey software. Data are presented as fold control and are representative of replicate experiments.

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