

Increased RhoA and RhoB Protein Accumulation in Cultured Human Trabecular Meshwork Cells by Lovastatin

Cynthia L. Von Zee,^{1,2} Michael P. Richards,¹ Ping Bu,³ Jay I. Perlman,^{3,4,5} and Evan B. Stubbs, Jr.^{2,3,6}

PURPOSE. This study aimed to determine the effect of lovastatin on Rho G-protein expression and activation in human trabecular meshwork (TM) cells.

METHODS. Confluent cultures of low-passage (primary) or transformed (GTM3) human TM cells were incubated overnight with vehicle (0.01% ethanol) or activated lovastatin (10 μ M). Changes in Rho mRNA, protein content, and activation were quantified by qRT-PCR, immunoblotting, and ELISA, respectively. F-actin organization was determined using Alexa Fluor 488-conjugated phalloidin.

RESULTS. Low-passage or transformed TM cells treated with lovastatin exhibited marked increases in RhoA and RhoB mRNA and protein content. Actinomycin D prevented lovastatin-dependent increases in RhoB, but not RhoA, protein accumulation. In contrast, cycloheximide prevented lovastatin from increasing both RhoA and RhoB. Supplementation with mevalonate or geranylgeranyl pyrophosphate prevented, whereas inhibition of geranylgeranyl transferase mimicked, the effects of lovastatin on RhoA and RhoB accumulation. The effect of lovastatin was dose dependent, with newly synthesized protein accumulating in the cytosol. The amount of functionally active (GTP-bound) RhoA in cell lysates was significantly reduced by lovastatin. Lovastatin altered the morphology of TM cells by disrupting F-actin organization.

CONCLUSIONS. Lovastatin enhances the accumulation of RhoA and RhoB in human TM cells, in part, by limiting geranylgeranyl isoprenylation of these G-proteins. We propose that post-translational geranylgeranylation serves as a regulator of both RhoA and RhoB protein expression and processing in human TM cells. Increased accumulation of unprenylated forms of RhoA and RhoB may disrupt Rho-dependent regulation of TM cell cytoskeletal organization. (*Invest Ophthalmol Vis Sci.* 2009;50:2816-2823) DOI:10.1167/iovs.08-2466

From ¹Research, ⁴Surgery, and ⁶Neurology Services, Department of Veterans Affairs, Edward Hines Jr. VA Hospital, Hines, Illinois; and the Departments of ³Ophthalmology, ⁵Pathology, and ²Cell Biology, Neurobiology, and Anatomy, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois.

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Corresponding author: Evan B. Stubbs, Jr., Research Service (151), 5th and Roosevelt, Edward Hines, Jr. VA Hospital, Hines, IL 60141; evan.stubbs@med.va.gov.

Statins are a group of potent 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that are widely used clinically as cholesterol-lowering agents.¹ Recently, statins have been reported to have beneficial effects that extend beyond their ability to reduce serum lipids. As a result, statins are now being aggressively evaluated as therapeutic agents for the management of a wide variety of disorders,²⁻⁴ including those affecting vision.^{5,6} Long-term statin therapy in patients with cardiovascular problems has been associated with a lower incidence and slowed progression of primary open angle glaucoma.⁵ Nagaoka et al.⁷ reported that systemic administration of simvastatin lowers intraocular pressure (IOP) in humans, whereas Song et al.⁸ found an increase in aqueous humor (AH) outflow in vitro after lovastatin treatment. Our own preliminary studies support a protective role of simvastatin during experimental ischemic-reperfusion retinal injury (Bu P, et al. *IOVS* 2007;48:ARVO E-Abstract 208). The mechanism(s) by which statins increase AH outflow, reduce IOP, and protect against retinal injury remain unclear.

HMG-CoA reductase is the rate-limiting enzyme in the mevalonate-cholesterol biosynthetic pathway.^{9,10} In addition to synthesizing cholesterol, the mevalonate pathway produces 15-carbon farnesyl (F) and 20-carbon geranylgeranyl (GG) pyrophosphate (PP) intermediates required for post-translational membrane targeting and functional activation of regulatory G-proteins, including Ras and Rho.^{11,12} Depletion of mevalonate by HMG-CoA reductase inhibition markedly limits the availability of FPP and GGPP isoprenoid intermediates.¹³ This limitation reduces farnesyl- and geranylgeranyl-transferase catalyzed isoprenylation of G-proteins.^{14,15} As a consequence, unprenylated forms of G-proteins have a reduced ability to associate with target membranes. Importantly, post-translationally immature forms of G-proteins may maintain partial function^{16,17} and interfere with the activity of mature membrane-anchored proteins.

Disruption of Rho G-protein mediated signaling events in trabecular meshwork (TM) cells may be of central importance in statin-dependent regulation of AH outflow. Rho G-proteins are a well-characterized family (RhoA-C, Rac1-3, and Cdc42) of small monomeric GTP binding proteins implicated in regulating a variety of TM cellular functions, including actin cytoskeletal organization and cell contractility.¹⁸ Inhibition of Rho with C3 exoenzyme, or expression of a dominant-negative mutant RhoA GTPase, in human eye anterior segments has been shown to increase AH outflow.^{19,20} The interaction of RhoA with Rho kinase effectors (ROCK1 [ROK β , p160ROCK] or ROCK2 [ROK α , Rho Kinase]) is believed to regulate actin polymerization,²¹⁻²³ making it a putative pharmacologic target for the regulation of AH outflow.²⁴ Both ROCK isoforms are expressed in human TM cells.^{25,26} Selective inhibitors of Rho kinases are currently being evaluated as novel therapeutic agents for the management of ocular hypertensive disorders.²⁷

Given the importance of the signaling pathways governed by Rho proteins in TM cells and their role in regulating AH outflow, we investigated in this study the consequence of

disrupting Rho isoprenylation on human TM cell function. Inhibition of HMG-CoA reductase with lovastatin elicits a marked and selective increase in RhoA and RhoB mRNA and G-protein content in human TM cells, in part, by limiting geranylgeranyl isoprenylation of these G-proteins (Von Zee CL, et al. *IOVS* 2008;49:ARVO E-Abstract:1628). We propose that post-translational geranylgeranylation facilitates RhoA and RhoB protein expression and processing in TM cells. We speculate that increased accumulation of unprenylated inactive (GDP-bound) forms of RhoA and RhoB may disrupt Rho-dependent regulation of TM cell cytoskeletal organization.

MATERIALS AND METHODS

Preparation and Culture of Human TM Cells

The use of human cadaver material in this study was approved by the Edward Hines, Jr. VA and Loyola University (Chicago, IL) institutional review boards. Fresh cadaver corneal rims were obtained (Illinois Eye Bank, Chicago, IL) at time of corneal transplant, and low-passage (primary) human TM cells were prepared using a collagenase-free procedure, as previously described.²⁸ TM tissue harvested from four separate donors were used to establish four separate low-passage TM cell lines. Individual TM cell lines were restricted to one to four passages. The purity of low-passage TM cell cultures typically exceeded 95%, as routinely determined by cell morphology (adherent flattened non-spindle shaped soma with prominent lamellipodia). Low-passage TM cell cultures exhibiting more than 5% spindle-shaped morphologic characteristics were considered contaminated with fibroblasts and discarded. Simian virus 40 (SV40)-transformed human TM cells from a male glaucomatous patient (GTM3) were a generous gift from A. F. Clark (Alcon Laboratories, Ft. Worth, TX).²⁹ Human low-passage TM cell cultures were established on cell culture flasks (Falcon Primaria; BD Biosciences, San Jose, CA) in Eagle's minimum essential medium containing 2 mM L-glutamine supplemented with 5% adult bovine serum, 10% fetal bovine serum, 0.1% gentamicin, 1% amphotericin B, and a mixture of essential (Invitrogen, Carlsbad, CA) and nonessential amino acids (Sigma Chemical Co., St. Louis, MO). Transformed human GTM3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM commercial glutamine-supplemented media (GlutaMAX-I; Invitrogen) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Low-passage and transformed cultures were maintained at 37°C under an atmosphere of 5% CO₂ and 95% air.

Lovastatin Activation and Treatment of Human TM Cell Cultures

Before use in cell culture, lovastatin was chemically activated by alkaline hydrolysis. Briefly, 4 mg of the inactive lactone prodrug was dissolved in 0.1 mL absolute ethanol and incubated for 2 hours at 50°C in the presence of 0.1 N NaOH. The resulting active open-ring hydroxy-acid form was neutralized (pH 7.2) with 0.1 N HCl and diluted to a final volume of 1 mL with sterile deionized water. Stock solutions of prepared lovastatin hydroxy acid (4 mg/mL) were stored at -20°C until use. Low-passage or transformed human TM cells were cultured to confluency and treated with vehicle (0.01% ethanol) or activated lovastatin (10 µM) for 24 hours⁸ unless noted otherwise. TM cell viability (>90%), determined by Trypan blue dye exclusion, was not affected by lovastatin treatment. To determine mechanism of action, cell cultures were co-treated with either L-mevalonate (5 mM), farnesyl pyrophosphate (10 µM), or geranylgeranyl pyrophosphate (10 µM). For transcription or translation experiments, cell cultures were pre-treated (1 hour, 37°C) with actinomycin D (0.5 µg/mL) or cycloheximide (1.4 µg/mL), respectively. To inhibit post-translational isoprenylation of Rho G-proteins, human TM cell cultures were incubated in the presence of selective farnesyl transferase (FTI-277, 10 µM) or geranylgeranyl transferase (GGTI-298, 10 µM) inhibitors.

Subcellular Fractionation

To determine the effect of lovastatin on Rho G-protein subcellular localization, lysates of human TM cells were prepared in deionized water supplemented with a commercial cocktail of protease inhibitors (Roche Applied Science, Indianapolis, IN). Particulate (crude membranes) and soluble (cytosolic) subcellular fractions were prepared as previously described.^{30,31} TM cell lysates were centrifuged at 100,000g for 30 minutes using an ultracentrifuge (Beckman Coulter, Fullerton, CA). The supernatant (soluble cytosolic fraction) was collected and stored at -80°C until use. The resultant membrane pellets were solubilized by gentle homogenization in 10 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS and centrifuged at 15,000g for 30 minutes to obtain a clarified supernatant. The resultant clarified membrane-soluble fraction was stored at -80°C until use. Protein concentrations in cell lysates (typically 7 to 8 mg/mL) and prepared subcellular fractions were measured by the BCA method (Pierce Biotechnology, Rockford, IL) using bovine serum albumin as the standard.

Immunoblot Analysis

Proteins (20 µg per lane) in cell lysates or subcellular fractions were resolved by SDS-PAGE and transferred onto nitrocellulose membranes as previously described.³² Washed membranes were blocked and incubated overnight at 4°C in the presence of a 1:1000 dilution of mouse anti-Rho (A,B,C) monoclonal (clone 55; Millipore, Temecula, CA). To determine which Rho isoforms were most affected by treatment, immunoblots were stripped and re-stained with Rho isoform-selective antibodies (1:200 dilution of mouse anti-RhoA monoclonal [clone 26C4] or rabbit anti-RhoB polyclonal [119]) from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoblots were similarly stripped and stained with a 1:10,000 dilution of rabbit anti-GAPDH polyclonal (Trevigen, Gaithersburg, MD) primary antibody as a protein loading control. Washed membranes were incubated for 1 hour at 23°C with a 1:2500 dilution of goat anti-mouse IgG or a 1:10,000 dilution of goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), respectively. Immunostained proteins were visualized by ECL. Relative changes in pan-Rho, RhoA, RhoB, or GAPDH total protein content was quantified by densitometry.

Rho Activation Assay

The levels of active GTP-bound RhoA protein in lysates prepared from lovastatin-treated cells was determined by ELISA using a commercially available G-LISA activation assay kit (BK124; Cytoskeleton Inc., Denver, CO). Briefly, cell lysates (135 µg protein) were incubated for 30 minutes at 4°C in microtiter wells pre-coated with an RBD domain of Rho-family effector proteins. Washed wells were incubated for 45 minutes at 23°C in the presence of a 1:250 dilution of mouse anti-RhoA antibody, followed by incubation with a 1:62.5 dilution of HRP-conjugated goat anti-mouse IgG secondary antibody, according to manufacturer's instructions. The level of captured GTP-bound (active) RhoA proteins present in cell lysates was quantified spectrophotometrically at 490 nm. The content of active GTP-bound RhoB proteins was not determined.

Real Time RT-PCR

Total RNA was extracted from low-passage or transformed human TM cells using reagent (TRIzol; Invitrogen) and 5 µg was reverse-transcribed (Super Script III First Strand Synthesis system; Invitrogen). RhoA-, RhoB-, and GAPDH-specific cDNA sequences were amplified by real-time (iQ SYBR Green Supermix; Bio-Rad, Hercules, CA) quantitative RT-PCR using a PCR detection system (Mini-Opticon; Bio-Rad). The following human-specific primer pairs were used: RhoA (forward, 5'-TGAAACTATGTGGCGGATATCG; reverse, 5'-TCTGCTTCTTCAGGTTTAACCGG) and RhoB (forward, 5'-AGGACTACGATCGTTACGCC; reverse, 5'-CAGCCATTCTGGGATCCGTAG). GAPDH (forward,

5'-TCCCTCAAGATTGTCAGCAA; reverse, 5-AGATCCACAACGGATACATT) primers were used as a reference control. Optimized amplification steps of 94°C × 5 minutes followed by 94°C × 45 seconds, 55°C × 60 seconds, and 72°C × 60 seconds for 40 cycles were used. For each sample, the specificity of the real-time reaction product was determined by melting curve analysis. Reaction efficiencies were typically more than 90%. The endogenous expression of GAPDH was unaltered by lovastatin, mevalonate, or lovastatin + mevalonate treatment. Relative fold-changes in gene expression in each sample were therefore normalized to expressed levels of GAPDH.

Filamentous Actin Organization in Human TM Cells

Human TM cells were grown to confluency on chambered coverslips (Nunc Lab-Tek; Thermo Fisher Scientific, Rochester, NY) and treated with vehicle or lovastatin, as described above. Cells were fixed for 15 minutes at 23°C by immersion in phosphate buffered (pH 7.4) 4% paraformaldehyde. Changes in F-actin organization were qualitatively determined by incubating fixed cells in the dark for 20 minutes at 23°C in the presence of 165 nM Alexa Fluor 488-conjugated phalloidin. F-actin stained cells were visualized by confocal microscopy.

Statistical Analysis

Data are expressed as the mean ± SEM unless noted otherwise. Statistical significance was determined by one-way ANOVA with a Dunnett's post hoc test. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Increase of Rho G-Protein Subfamily Content in Human TM Cells by Lovastatin

Transformed (GTM3) human TM cells cultured in the presence of activated lovastatin expressed marked increases (more than twofold) in the steady state level of Rho proteins (Fig. 1). RhoA was found to be expressed constitutively in vehicle-treated GTM3 cells and increased 12-fold with lovastatin treatment (Fig. 1, RhoA). By comparison, GTM3 cells at rest did not express measurable levels of total RhoB G-proteins. Similar to RhoA, however, lovastatin treatment elicited a marked increase (54-fold over background) in RhoB content (Fig. 1, RhoB). Lovastatin-dependent changes in protein expression were selective for the Rho subfamily of G-proteins. Expression of other Rho family members (Rac1 or Cdc42) in resting GTM3 cells was below the level of detection and remained unaltered after lovastatin treatment (data not shown).

Co-treatment of GTM3 cell cultures with mevalonate, the immediate metabolic product of HMG-CoA reductase, completely prevented lovastatin-dependent increases in RhoA and RhoB (Fig. 1). Mevalonate treatment alone had no effect on Rho or GAPDH accumulation (Fig. 1). The content of RhoC proteins present in GTM3 cells was not addressed.

Lovastatin-dependent changes in Rho content were not unique to transformed TM cells. Low-passage human TM cells treated with lovastatin exhibited marked (>90-fold above background) increases in the content of RhoA and RhoB that were prevented by co-incubation with mevalonate (Fig. 2). By comparison, RhoA and RhoB expression in vehicle-treated low-passage human TM cells remained below the level of detection despite measurable quantities of GAPDH (Fig. 2).

Alteration of Rho G-Protein Subcellular Localization and Functional Activation by Lovastatin

In resting GTM3 cells, Rho was found to be predominantly associated within cell membranes with little expressed in cell

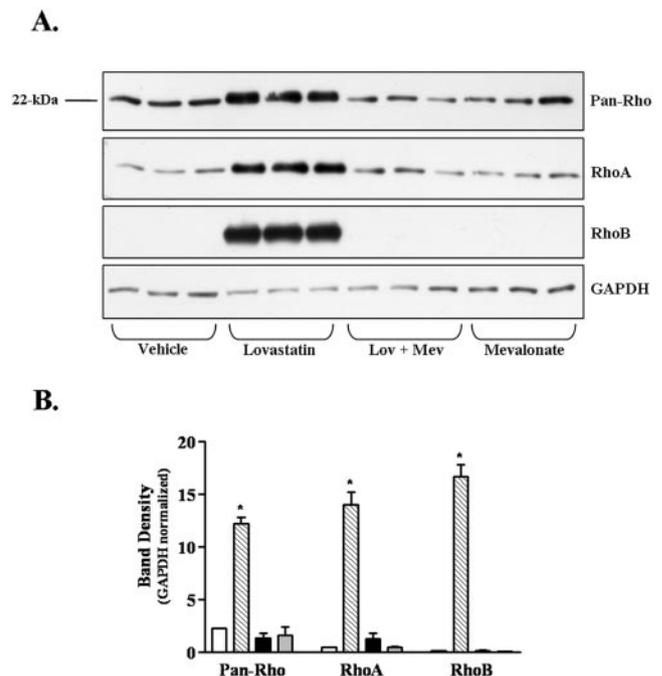


FIGURE 1. Lovastatin increases Rho protein content in human GTM3 cells. Confluent GTM3 cells were incubated (24 hours) with vehicle (0.01% ethanol), lovastatin (Lov, 10 μ M), mevalonate (Mev, 5 mM), or a combination of the two (Lov + Mev) and subsequently lysed in the presence of protease inhibitors. Proteins in cell lysates (20 μ g/lane) were resolved on 4–20% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunostained for the presence of Rho subfamily of G-proteins. (A) Western immunoblot of pan-Rho, RhoA, and RhoB protein expressed in cell lysates from a single experiment performed in triplicate. Levels of GAPDH, a housekeeping enzyme, are shown for comparison as loading controls. (B) Quantitative densitometric comparison of the results shown in (A) normalized to GAPDH and expressed as the mean ± SD. Vehicle (open bar), lovastatin (hatched bar), lovastatin + mevalonate (solid bar), mevalonate (gray bar). * $P < 0.01$ (one-way ANOVA with Dunnett's post hoc analysis).

cytosol (Fig. 3). Lovastatin elicited a dose-dependent decrease in membrane-associated Rho proteins, with a disproportionate accumulation of both RhoA and RhoB in the cell cytosol (Fig. 3). Notable increases in soluble RhoA were observed beginning at ~100 nM lovastatin with a half-maximal effectiveness of 400 nM. By comparison, higher concentrations of lovastatin (>1 μ M) were needed to alter soluble RhoB content in these cells. At 1 μ M lovastatin, a lightly immunostained RhoB doublet was appreciated, the significance of which remains unclear. Membrane-associated RhoB G-proteins were not detectable in vehicle-treated cells.

By altering the subcellular localization of Rho, lovastatin may influence the amount of functionally active (GTP-bound) Rho G-proteins present in GTM3 cells. Measurable quantities of GTP-bound RhoA were present in vehicle-treated cells (Fig. 4). Acute (30 minutes) lovastatin (10 μ M) treatment of GTM3 cells caused a modest (~20%) but significant ($P < 0.01$) reduction in the level of GTP-bound RhoA. At 24 hours, when lovastatin is seen to increase the content of total soluble RhoA, functionally active (GTP-bound) RhoA remained significantly ($P < 0.001$) reduced compared to vehicle-treated controls (Fig. 4).

Effect of Lovastatin on Rho G-Protein mRNA Content

The relative content of Rho isoform-specific mRNA in vehicle- or lovastatin-treated TM cells is shown in Table 1. Constitutive

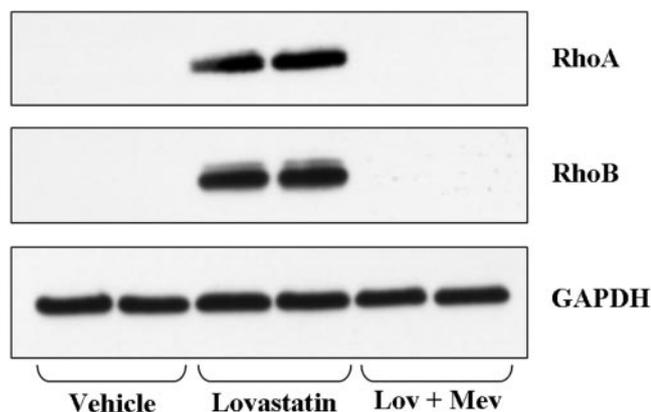


FIGURE 2. Lovastatin increases Rho protein content in low-passage human TM cells. Low-passage human TM cells were harvested from discarded corneoscleral rims and confluent cultures were treated for 48 hours with vehicle (0.01% ethanol), lovastatin (10 μ M), or with lovastatin plus 5 mM mevalonate (Lov + Mev). Cell lysates were prepared and protein (18 μ g/lane) resolved as described in Figure 1. Shown is a representative Western immunoblot of RhoA and RhoB protein expression from cells harvested from one to three different donor preparations. GAPDH content is shown for comparison as a loading control.

levels of RhoA-specific and RhoB-specific mRNA expressed in GTM3 cells were increased twofold and 48-fold, respectively, by lovastatin (Table 1). Co-incubation with mevalonate (5 mM) prevented lovastatin-dependent changes in RhoA or RhoB mRNA expression (Table 1). Lovastatin treatment of low-passage human TM cells elicited similar increases in both RhoA (twofold) and RhoB (sevenfold) mRNA content (Table 1).

Effect of Lovastatin on Transcriptional and Translational Regulation of Rho G-Protein Expression

Lovastatin increases in RhoA content in GTM3 cells were not affected by pretreatment (0.5 μ g/mL, 1 hour) with actinomycin D (Fig. 5), an inhibitor of gene transcription. In contrast, actinomycin D ablated lovastatin-dependent increases in RhoB expression (Fig. 5). The constitutive expression of GAPDH was unaltered by actinomycin D. Inhibiting de novo protein syn-

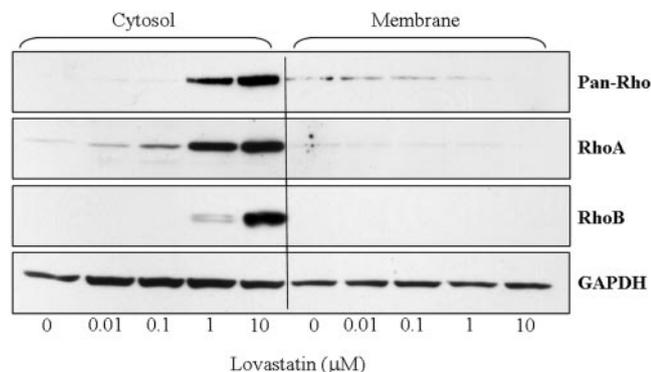


FIGURE 3. Lovastatin elicits RhoA and RhoB protein accumulation in the cytosol. Confluent GTM3 cells were incubated in the presence of lovastatin as indicated (0–10 μ M; 24 hours) and soluble (cytosol) and particulate (membrane) fractions prepared. Shown is a representative Western immunoblot of pan-Rho, RhoA, and RhoB protein expression in subcellular fractions (20 μ g/lane) prepared from three separate experiments. GAPDH content is shown for comparison as a loading control.

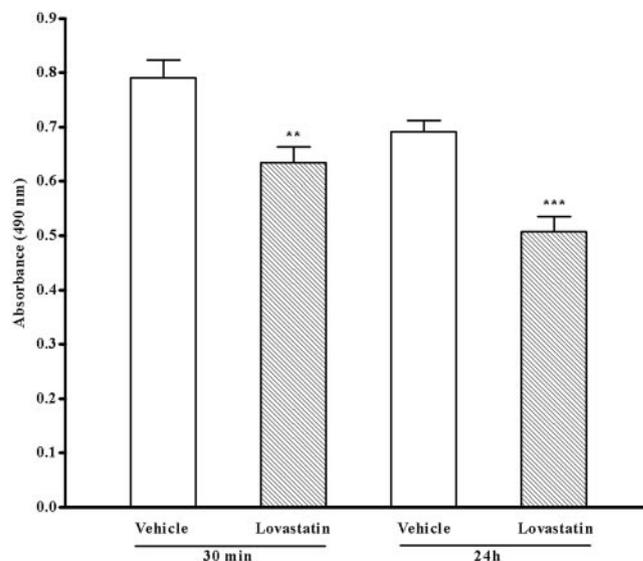


FIGURE 4. Lovastatin decreases GTP-bound RhoA content in GTM3 cells. Confluent GTM3 cells were incubated with vehicle (0.01% ethanol) or lovastatin (10 μ M) as indicated and the content of GTP-bound RhoA in whole cell lysates was determined by G-LISA. Data shown are expressed as the mean \pm SD from a single experiment performed in triplicate. ** $P < 0.01$; *** $P < 0.001$ compared with vehicle control (one-way ANOVA with Dunnett's post hoc analysis).

thesis with cycloheximide (1.4 μ g/mL, 1 hour pretreatment) blocked lovastatin-dependent increases in both RhoA and RhoB protein expression (Fig. 6).

Increase of Rho Content by Inhibiting Protein Geranylgeranylation with Lovastatin

Supplementing GTM3 cell cultures with geranylgeranyl pyrophosphate, but not farnesyl pyrophosphate, prevented lovastatin increases in RhoA and RhoB content (Fig. 7). Lovastatin-dependent increases in RhoA and RhoB content in low-passage human TM cells were similarly prevented by GGPP supplementation (Fig. 8).

The mechanism by which geranylgeranyl isoprenoid supplementation affects lovastatin-dependent Rho G-protein expression was determined by treating GTM3 cell cultures with specific inhibitors of farnesyl transferase (FTI-277) or geranylgeranyl transferase-I (GGTI-298). The content of RhoA and RhoB in FTI-277 treated GTM3 cells was similar to vehicle-treated controls (Fig. 9). In contrast, GTM3 cells treated with GGTI-298 exhibited a significant increase in RhoA and RhoB content, mimicking the effect of lovastatin. Confluent cultures of low-passage human TM cells treated with GGTI-298 similarly exhibited increases in RhoA and RhoB content (Fig. 10). The effect of lovastatin on the levels of FPP or GGPP in low-passage or transformed human TM cells was not determined.

Functional Disruption of Filamentous Actin Organization by Lovastatin

Transformed GTM3 cells grown to confluency exhibit prominent phalloidin-positive stress fiber organization (Fig. 11). Cells treated with lovastatin showed marked changes in morphology including a loss of phalloidin-positive stress fiber staining (Fig. 11B). Supplementation with geranylgeranyl pyrophosphates prevented lovastatin-dependent changes in cell morphology and in phalloidin-positive stress fiber staining (Fig. 11C), whereas treating cells with GGTI-298 (10 μ M) qualitatively mimicked the effect of lovastatin (Fig. 11D). Low-passage hu-

TABLE 1. Lovastatin Increases Rho mRNA Content in TM Cells

TM Cell Type	Treatment	RhoA mRNA Levels	RhoB mRNA Levels
Transformed (GTM3)	Vehicle	1.0 ± 0.3	1.0 ± 0.4
	Lovastatin	2.3 ± 0.6*	47.8 ± 12.4**
	Mevalonate	1.1 ± 0.4	0.9 ± 0.6
	Lovastatin + Mevalonate	1.4 ± 0.2	3.1 ± 3.3
Low Passage	Vehicle	1.0	1.0
	Lovastatin	2.2	6.8

Transformed (GTM3, $n = 3$) or low passage (primary, $n = 2$) human TM cells were incubated (24–48 hours) in the presence of vehicle (0.01% ethanol) or lovastatin (10 μ M), and RhoA-specific or RhoB-specific mRNA content was determined by qRT-PCR. Data shown are mean \pm SD (transformed) or average (low passage) fold-changes expressed relative to GAPDH-specific mRNA content. * $P < 0.05$; ** $P < 0.01$ (one-way ANOVA with Dunnett's post hoc analysis).

man TM cell cultures also displayed prominent phalloidin-positive stress fiber organization (Fig. 12A). Similar to GTM3 cells, prominent changes in human TM cell morphology and stress fiber organization were evident after 48 hours of lovastatin treatment (Fig. 12B) and were preventable by co-incubation with mevalonate (Fig. 12C).

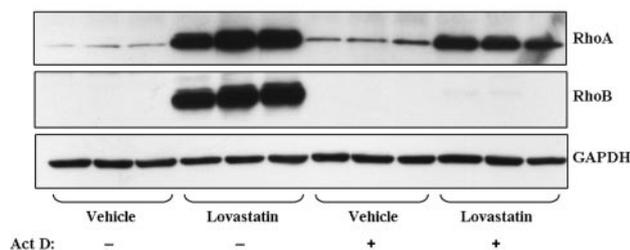
DISCUSSION

In this study, we report the novel finding that lovastatin elicits a marked and selective increase in the content of RhoA and RhoB G-proteins in low-passage (primary) and transformed (GTM3) human trabecular meshwork (TM) cells. Inhibition of HMG-CoA reductase with lovastatin leads to enhanced accumulation of RhoA and RhoB, in part, by limiting geranylgeranyl

isoprenylation of these G-proteins. We propose that post-translational geranylgeranylation facilitates RhoA and RhoB protein expression and processing in human TM cells. We speculate that increased accumulation of unprenylated inactive (GDP-bound) forms of RhoA and RhoB may disrupt Rho-dependent regulation of TM cell cytoskeletal organization.

Disruption of Rho G-protein mediated signaling events in TM cells may be of central importance in statin-dependent regulation of AH outflow. The mechanism by which statins increase AH outflow⁸ and reduce IOP⁷ remains unclear. Competitive inhibition of HMG-CoA reductase with mevastatin triggers an adaptive response in cultured mammalian cells that results in a marked 200-fold increase in the expression of the reductase protein.^{33,34} Upregulation of reductase expression by lovastatin involves several levels of control including induction of transcription, enhanced mRNA translation, and slowing of reductase degradation,³³ consistent with classical feedback-

A.



B.

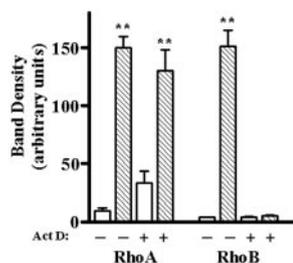
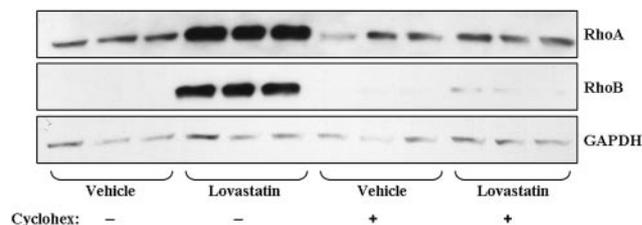


FIGURE 5. Actinomycin D inhibits lovastatin-dependent increases in RhoA and RhoB protein content. Confluent GTM3 cells were pre-treated (1 hour) with (+) or without (-) 0.5 μ g/mL actinomycin D (Act D) and incubated for an additional 24 hours with vehicle (0.01% ethanol) or lovastatin (10 μ M) as indicated. (A) Western immunoblot of RhoA and RhoB protein expressed in cell lysates (20 μ g protein/lane) prepared from a single experiment performed in triplicate. GAPDH content is shown for comparison as a loading control. (B) Quantitative densitometric comparison of the results shown in (A) expressed as the mean \pm SD. Vehicle (open bar), lovastatin (hatched bar). ** $P < 0.01$ (one-way ANOVA with Dunnett's post hoc analysis).

A.



B.

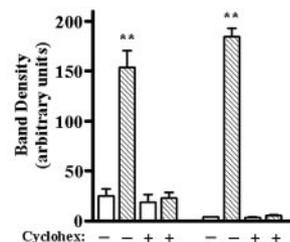


FIGURE 6. Cycloheximide prevents lovastatin increases in RhoA and RhoB protein content. Confluent GTM3 cells were pre-treated (1 hour) with (+) or without (-) 1.4 μ g/mL cycloheximide (Cyclohex) and incubated for an additional 24 hours with vehicle (0.01% ethanol) or lovastatin (10 μ M) as indicated. (A) Western immunoblot of RhoA and RhoB protein expressed in cell lysates (20 μ g protein/lane) prepared from a single experiment performed in triplicate. GAPDH content is shown for comparison as a loading control. (B) Quantitative densitometric comparison of the results shown in (A) expressed as the mean \pm SD. Vehicle (open bar), lovastatin (hatched bar). ** $P < 0.01$ (one-way ANOVA with Dunnett's post hoc analysis).

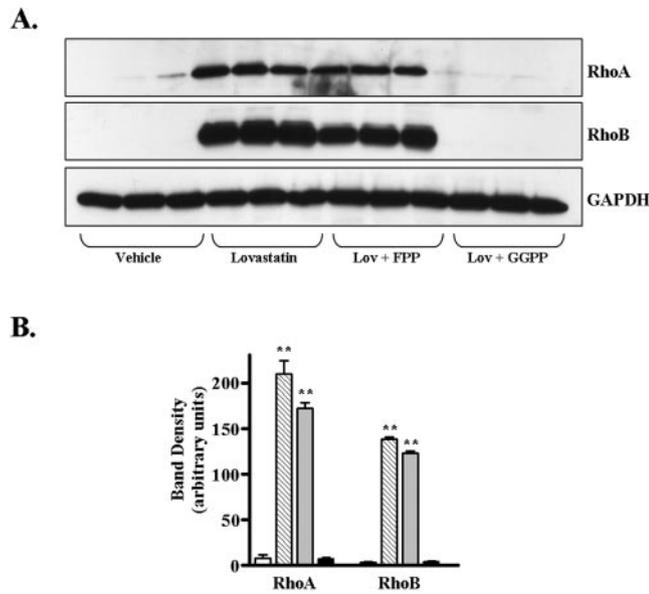


FIGURE 7. Geranylgeranyl, but not farnesyl, pyrophosphate prevents lovastatin increases in RhoA and RhoB protein content. Confluent GTM3 cells were incubated (24 hours) with vehicle (0.01% ethanol), lovastatin (10 μ M), lovastatin plus farnesyl-PP (Lov + FPP, 10 μ M each) or lovastatin plus geranylgeranyl-PP (Lov + GGPP, 10 μ M each) as indicated. (A) Western immunoblot of RhoA and RhoB protein expressed in cell lysates (20 μ g protein/lane) prepared from a single experiment performed in triplicate representative of two separate experiments. GAPDH content is shown for comparison as a loading control. (B) Quantitative densitometric comparison of the results shown in (A) expressed as the mean \pm SD. Vehicle (open bar), lovastatin (hatched bar), lovastatin + farnesyl-PP (gray bar), lovastatin + geranylgeranyl-PP (solid bar). ** P < 0.01 (one-way ANOVA with Dunnett's post hoc analysis).

regulatory control of a rate-limiting enzyme. However, more recent evidence suggests the regulation of protein expression by lovastatin may not be uniquely restricted to HMG-CoA

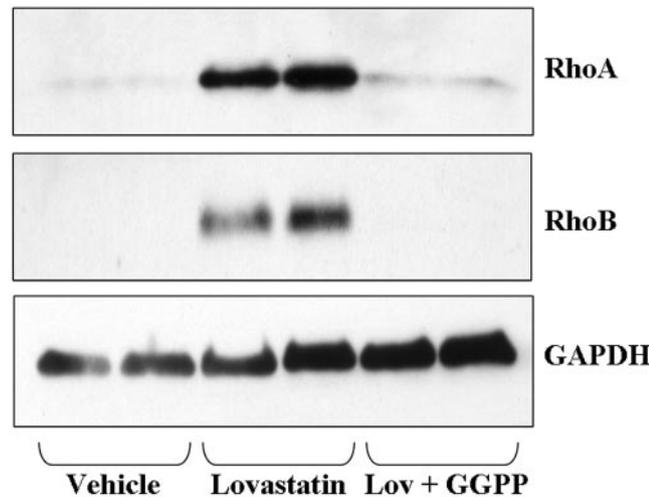


FIGURE 8. Geranylgeranyl pyrophosphate supplementation prevents lovastatin increases in RhoA and RhoB protein content in low-passage human TM cells. Confluent cultures of low-passage human TM cells were incubated (48 hours) with vehicle (0.01% ethanol), lovastatin (10 μ M), or lovastatin plus geranylgeranyl-PP (Lov + GGPP, 10 μ M each) as indicated. Western immunoblot of RhoA and RhoB protein expressed in cell lysates (10 μ g protein/lane) prepared from a single experiment performed in duplicate. GAPDH content is shown for comparison as a loading control.

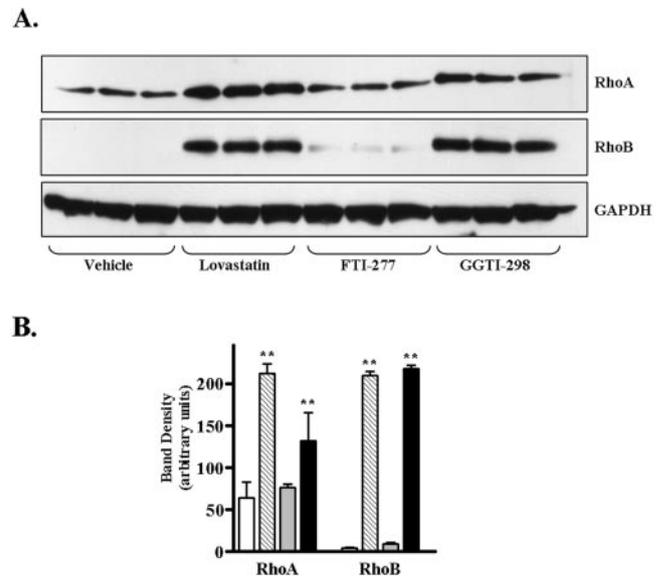


FIGURE 9. Inhibition of geranylgeranyl transferase, but not farnesyl transferase, increases RhoA and RhoB protein content. Confluent GTM3 cells were incubated (24 hours) with vehicle (0.01% ethanol), lovastatin (10 μ M), farnesyl transferase inhibitor (FTI-277, 10 μ M), or geranylgeranyl transferase inhibitor (GGTI-298, 10 μ M) as indicated. (A) Western immunoblot of RhoA and RhoB protein expressed in cell lysates (20 μ g protein/lane) prepared from two separate experiments each performed in triplicate. GAPDH content is shown for comparison as a loading control. (B) Quantitative densitometric comparison of the results shown in (A) expressed as the mean \pm SD. Vehicle (open bar), lovastatin (hatched bar), FTI-277 (gray bar), GGTI-298 (solid bar). ** P < 0.01 (one-way ANOVA with Dunnett's post hoc analysis).

reductase. Holstein et al. reported that HMG-CoA reductase inhibition leads to upregulation of additional proteins modified by the mevalonate-cholesterol pathway, including members of the Rho subfamily of G-proteins.³⁵ Our own experience working with HCT116 colon carcinoma cells confirms the ability of statins to increase Rho protein expression (Deadwyler GD,

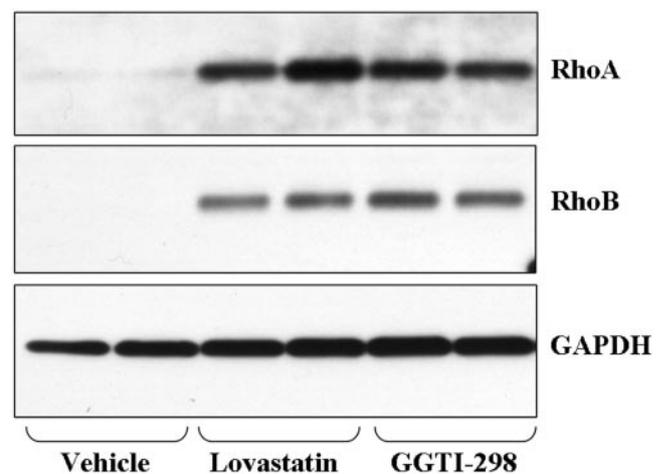


FIGURE 10. Inhibition of geranylgeranyl transferase increases RhoA and RhoB protein content in low-passage human TM cells. Confluent cultures of low-passage human TM cells were incubated (48 hours) with vehicle (0.01% ethanol), lovastatin (10 μ M), or geranylgeranyl transferase inhibitor (GGTI-298, 10 μ M) as indicated. Western immunoblot of RhoA and RhoB protein expressed in cell lysates (10 μ g protein/lane) prepared from a single experiment performed in duplicate. GAPDH content is shown for comparison as a loading control.

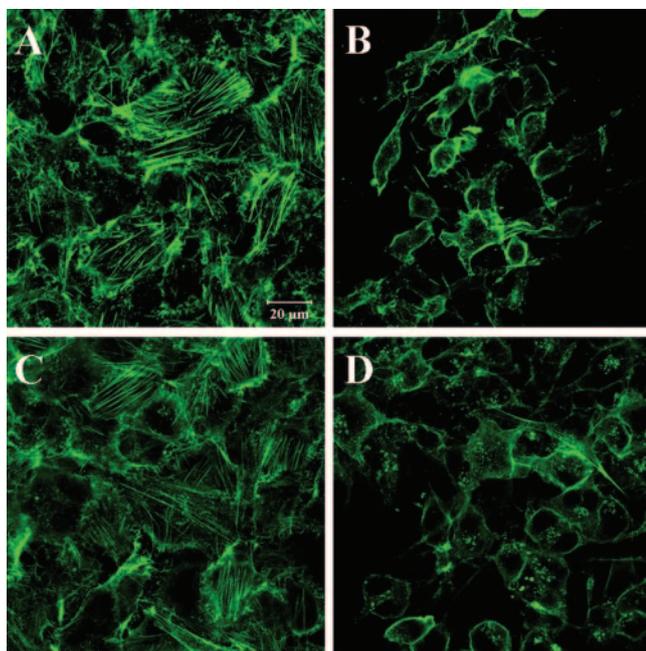


FIGURE 11. Lovastatin disrupts filamentous actin organization. GTM3 cells cultured in chambered coverslips were incubated (24 hours) with (A) vehicle (0.01% ethanol), (B) lovastatin (10 μ M), (C) lovastatin plus geranylgeranyl-PP (10 μ M each), or (D) geranylgeranyl transferase inhibitor (GGTI-298, 10 μ M), and subsequently stained with Alexa Fluor 488-conjugated phalloidin. Shown are confocal photomicrographs of stained cultures representative of 2 to 12 separate coverslips. Bar, 20 μ m.

Stubbs EB Jr, unpublished observation, 2006). Statin-dependent changes in Rho expression, however, are not unique to transformed cells. As shown in this study, lovastatin elicits a prominent accumulation of RhoA and RhoB protein in low-passage human TM cells. Importantly, other members of the Rho G-protein family (Rac1 or Cdc42, data not shown) were not affected by lovastatin treatment.

Post-translational isoprenylation facilitates membrane targeting and functional activation of newly synthesized Rho proteins.¹¹ Limiting the availability of essential FPP and GGPP metabolic intermediates by HMG-CoA reductase inhibition severely impairs protein isoprenylation.¹³ Restoration of protein isoprenylation by the addition of mevalonate prevents lovastatin increases in Rho protein expression. One anticipated consequence of impaired protein isoprenylation is the measurable loss of membrane-associated Rho, possibly resulting in an increased accumulation of soluble unprenylated protein. However, the disproportionate accumulation of RhoA and RhoB proteins in the cytosol of lovastatin treated cells observed here, we argue, is suggestive of a more complex mechanism regu-

lating Rho expression. Holstein et al.³⁵ proposed a role of isoprenoid pyrophosphates as active repressors of Rho G-protein expression. Consistent with this thesis, supplementation of TM cell cultures with GGPP, but not FPP, prevented lovastatin increases in both RhoA and RhoB. Rho subfamily of G-proteins are modified largely by transferase-catalyzed geranylgeranylation, which may explain the isoprenoid selectivity.^{14,15}

Whereas isoprenoid pyrophosphates may act as repressors of Rho expression, isoprenylation itself may target Rho proteins for degradation.³⁶ Under conditions where isoprenoid availability was not limited, we found that selective inhibition of geranylgeranyl transferase markedly increases Rho protein expression, similar to that seen for lovastatin. Post-translational geranylgeranylation may serve to facilitate Rho protein degradation in human TM cells.

The mevalonate-cholesterol pathway may also regulate transcriptional and translational expression of Rho. Statin depletion of mevalonate reportedly increases RhoB mRNA.^{35,36} We observed, in a mevalonate-preventable manner, lovastatin increases in RhoA mRNA and RhoB mRNA. By inhibiting transcription, we were able to prevent lovastatin from increasing RhoB, but not RhoA, protein accumulation. By comparison, inhibiting de novo protein synthesis with cycloheximide prevented lovastatin from increasing RhoA and RhoB protein. These findings collectively support a negative role of isoprenoid metabolic intermediates or isoprenylated proteins themselves on RhoB promoter activity.³⁷ The regulation of RhoA expression by lovastatin in TM cells most likely occurs by a post-transcriptional mechanism, possibly by enhancing RhoA mRNA stability.

The functional relevance of these findings is underscored by studies showing that unprenylated forms of G-proteins are not biologically inert.^{16,17} Interference of Ras-signaling by functionally active unprenylated Ras G-proteins suggest functional consequences.^{38,39} However, the majority of soluble unprenylated Rho proteins that accumulate in lovastatin-treated TM cells did not appear to be functionally active (GTP-bound). TM cells treated with lovastatin or geranylgeranyl transferase inhibitor exhibited decreased actin stress fiber organization coincident with increased unprenylated Rho protein accumulation. Isoprenoid supplementation prevented lovastatin dissociation of filamentous actin. It remains to be elucidated whether increased stability of unprenylated inactive (GDP-bound) Rho proteins leads to changes in Rho signaling.

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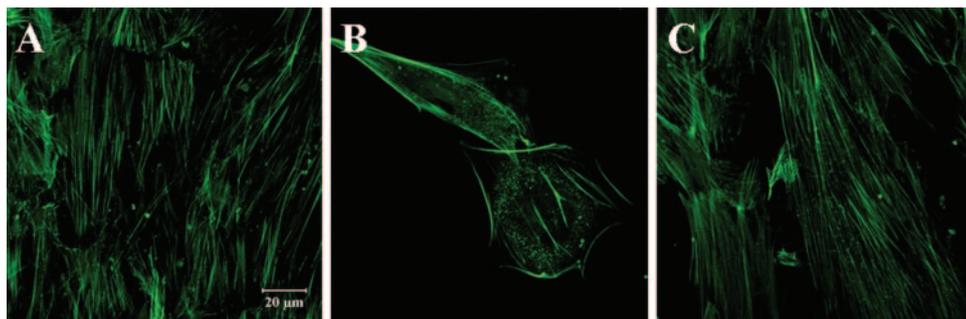


FIGURE 12. Lovastatin disrupts filamentous actin organization in low-passage human TM cells. Low-passage human TM cells cultured in chambered coverslips were incubated (48 hours) with (A) vehicle (0.01% ethanol), (B) lovastatin (10 μ M), or (C) lovastatin (10 μ M) plus mevalonate (5 mM), and subsequently stained with Alexa Fluor 488-conjugated phalloidin. Shown are confocal photomicrographs of stained cultures representative of two to eight separate coverslips. Bar, 20 μ m.

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