Lovastatin-Induced Cytoskeletal Reorganization in Lens Epithelial Cells: Role of Rho GTPases

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PURPOSE. To understand the involvement of isoprenylated small guanosine triphosphatases (GTPases) in lovastatin-induced cataractogenesis, Rho- and Rac-mediated cell adhesion and actin cytoskeletal reorganization were investigated in lovastatin-treated lens epithelial cells.

METHODS. The effects of lovastatin on F-actin reorganization (phalloidin staining), focal adhesion formation (paxillin or vinculin), cell–cell adhesions (cadherin and β-catenin), and protein tyrosine phosphorylation were evaluated in human and porcine lens epithelial cells by immunocytochemical staining with specific antibodies. To explore the involvement of the Rho and Rac GTPases in lovastatin-mediated effects, changes in distribution of Rho and Rac GTPases were analyzed by Western blot analysis, and the effects of C3-exoenzyme on lovastatin-induced cytoskeletal changes were evaluated by immunocytochemical analysis.

RESULTS. Lovastatin induced drastic changes in cell shape in both human and porcine lens epithelial cells, including a profound loss of actin stress fibers, focal adhesions, protein phosphoryrosine, and cell–cell adhesions. Lovastatin treatment also led to the accumulation of nonisoprenylated Rho and Rac GTPases in cytosolic fraction. Supplementation of culture media with geranylgeranyl pyrophosphate dramatically reversed the lovastatin-induced morphologic and cytoskeletal changes, whereas farnesyl pyrophosphate was ineffective. Treatment of cells with C3-exoenzyme (a Rho GTPase-specific inhibitor), however, abolished the geranylgeranyl-supplementation-induced recovery from the morphologic and cytoskeletal effects of lovastatin.

CONCLUSIONS. This study demonstrates that inhibition of protein prenylation by lovastatin leads to disruption of actin cytoskeletal organization, and to loss of integrin-mediated focal adhesions and cadherin-mediated cell–cell adhesions in lens epithelial cells. Based on isoprenoid supplementation studies, it could be concluded that impairment of geranylgeranylated Rho and Rac GTPase function is most likely responsible for lovastatin-induced cytoskeletal changes in lens epithelial cells. (Invest Ophthalmol Vis Sci. 2001;42:2610–2615)

Materials and Methods

Cell Cultures

The human lens epithelial cells (cell line, SRA 01/04)16 were cultured in DMEM containing 20% fetal bovine serum (FBS) and 20 µg/ml gentamicin (Gibco BRL Life Technologies, Gaithersburg, MD), and supported by Grant EY12201 from the National Institutes of Health (PVR).

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porcine lens primary epithelial cell cultures were isolated by collagenase digestion. Samples of lens epithelium obtained from freshly enucleated porcine eyes from a local abattoir were digested with collagenase type 4 (1.5 mg/ml; Worthington Biochemical Corp., Lakewood, NJ) for 2 hours at 37°C, in medium (T-199; Gibco BRL Life Technologies) containing 10 mg/ml porcine albumin (Sigma-Aldrich, St. Louis, MO). After digestion, samples were spun down at 3000 rpm and plated on 2% gelatin-coated petri dishes (BD Biosciences, Bedford, MA) and cultured with DMEM, 20% FBS, and gentamicin. Cells derived from the third passage were typically used in these studies.

Staining for Cytoskeletal Proteins

Cell cultures were grown to confluence on glass coverslips coated with 2% gelatin in complete medium containing 10% fetal calf serum, and treated with 20 μM (human cell line) or 30 μM (porcine primary cultures) lovastatin (a gift from Merck Pharmaceuticals, Rahway, NJ) for periods of 18 and 24 hours, respectively. Lovastatin-induced morphologic changes were monitored by phase-contrast microscopy (IM 35; Carl Zeiss, Thornwood, NY). After drug treatment, cells were fixed at room temperature for 10 minutes in 5.7% formaldehyde (vol/vol) in phosphate-buffered saline (PBS). They were then washed with cytoskeletal buffer (10 mM 2-[N-morpholino]ethane sulfonic acid (MES) containing 150 mM NaCl, 5 mM EGTA, 5 mM MgCl2, and 5 mM glucose [pH 6.1]), followed by permeabilization for 10 minutes with 0.1% Triton X-100 in PBS and blocking with serum-containing buffer (10% FBS in PBS with 0.02% sodium azide).

Cytoskeletal staining was performed as described previously.17 Briefly, for F-actin staining, cells were incubated with tetrarhodamine isothiocyanate (TRITC)-Phalloidin (500 ng/ml; Sigma-Aldrich) in serum containing buffer with 0.2% saponin for 45 minutes. For detection of focal adhesions, cells were immunostained, either with monoclonal anti-paxillin or anti-vinculin (Transduction Laboratories, Lexington, KY, and Sigma Chemical Co., respectively), and protein phosphotyrosine, cadherins, and β-catenin were immunostained for 2 hours at room temperature with monoclonal anti-phosphotyrosine, polyclonal anti-paxillin, and polyclonal anti-β-catenin antibodies (all from Sigma Chemical Co.) in serum containing buffer with 0.2% saponin. The secondary antibodies used in all cases were conjugated with TRITC (Sigma-Aldrich). Coverslips were mounted on glass slides with Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL), and micrographs were obtained with a fluorescence microscope (Axioplan-2; Zeiss).

Western Blot Analysis

For the preparation of cell lysates, cells were grown to confluence in 60-mm petri dishes and then treated with lovastatin (30 μM) for 18 hours. The cells were washed twice with PBS, scraped into cold PBS, and pelleted. Cells were sonicated in 0.5 ml cell lysis buffer (20 mM Tris [pH 7.4] containing 0.5 mM sodium orthovanadate, 0.2 mM EDTA, 10 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 M NaCl, 50 mM NaF, 25 μg/ml each of aprotonin and leupeptin, and 1 μM okadaic acid). After removal of the insoluble fraction (by centrifugation at 20,000×g for 15 minutes), protein content of the supernatants was determined by the Bradford method,18 using a commercial protein assay reagent (Bio-Rad, Richmond, CA).

SDS-polyacrylamide gel electrophoresis was performed with gels containing 12.5% polyacrylamide. Proteins were transferred to nitrocellulose filters, as described previously,17 and Western blot analysis was performed with polyclonal antibodies raised against RhoA and Rac1 (Santa Cruz Biotechnology, Santa Cruz, CA) and peroxidase-coupled secondary antibodies in conjunction with detection by enhanced chemiluminescence (Amer sham Pharmacia Biotechnology, Piscataway, NJ).

Isoprenoid Supplementation Studies

After treating the cells with lovastatin (20 μM) in 10% serum-containing medium for 18 hours, 10 μM of either geranylgeranyl pyrophosphate or farnesyl pyrophosphate (Sigma-Aldrich) was added to the lovastatin-containing medium. Human lens epithelial cells and primary cultures of porcine lens epithelial cells were maintained in supplemented medium for 4 and 24 hours, respectively. At the end of the supplementation period, cells were monitored for reversal of lovastatin-induced morphologic changes. Cells from these experiments were subsequently fixed and stained for actin, focal adhesions, and tyrosine phosphorylated proteins, as described earlier.

In another set of supplementation experiments, human lens epithelial cells grown on gelatin-coated glass coverslips were pretreated with 10 μg/ml C3-exoenzyme (an irreversible inhibitor of Rho GTPase) for 48 hours in complete medium containing 10% serum, to inactivate the Rho GTPases. Lovastatin (20 μM) was then added to the medium, and incubation continued for 18 hours, before supplementation of the culture medium with geranylgeranyl pyrophosphate for 8 hours. Finally, cells were fixed and stained for actin stress fibers to check for the involvement of Rho GTPase in lovastatin-mediated effects. To determine the effect of geranylgeranyl pyrophosphate supplementation on the distribution of Rho and Rac GTPases, lovastatin-treated cells were supplemented with geranylgeranyl pyrophosphate (10 μM) for 8 hours, and cell lysates were prepared for Western blot analysis as described earlier.

Results

Lovastatin-Induced Morphologic and Cytoskeletal Changes in Lens Epithelial Cells

Lovastatin treatment induced dramatic morphologic alterations in confluent cultures of both the human lens epithelial cell line (SRA 01/04) and porcine lens-derived primary cell cultures. Exposure to lovastatin caused cell rounding and cell–cell separation and induced the formation of filamentous extensions and processes in both the cell types. The human lens epithelial cell line was found to be more sensitive to lovastatin, whereas porcine primary lens epithelial cells required a higher concentration of lovastatin applied over a longer duration to induce morphologic changes similar to those observed in the human lens epithelial cell line (data not shown for porcine primary lens cells, but representative changes for human cell line can be seen in Fig. 4).

Phalloidin staining for actin stress fibers in confluent cultures of human lens epithelial cells grown on gelatin-coated coverslips revealed the presence of regular hexagonal cortical actin stress fibers in control cells (Fig. 1). In contrast, lovastatin treatment (20 μM for 18 hours) of human lens epithelial cells resulted in nearly complete loss of actin stress fibers. Similar changes in actin fibers were also evident in porcine lens epithelial cell primary cultures treated with lovastatin (30 μM for 24 hours; data not shown). Compared with human cells, actin stress fibers of porcine primary cultures stained very intensely and appeared to be organized in a transverse plane throughout the cell body.

Both human and porcine cells stained strongly for integrin-mediated focal adhesions (detected by staining for vinculin or paxillin) and protein phosphotyrosine. Lovastatin treatment significantly decreased focal adhesions in both cell types (Fig. 1). Phosphotyrosine labeling was also reduced markedly in response to lovastatin treatment (Fig. 1). The human lens epithelial cell line displayed a clear pattern of cadherin-catenin–mediated cell–cell adhesions, localized especially at the intercellular junctions in a “zip-lock” pattern, as evident from staining with pan-cadherin and β-catenin antibodies (Fig. 2). Lovastatin treatment (20 μM for 18 hours) was found to cause a marked reduction in staining for both cadherin and β-catenin, indicating a loss of cell–cell adhesions (Fig. 2).

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Changes in the Distribution of RhoA and Rac1 in Lovastatin-Treated Lens Epithelial Cells

To explore whether the lovastatin-induced cytoskeletal changes are associated with reduction in RhoA and Rac1 isoprenylation and activity, we analyzed the distribution profiles of RhoA and Rac1 in the soluble fraction of untreated (control), lovastatin-treated, or lovastatin-treated and geranylgeranyl-supplemented lens epithelial cells. While RhoA was easily detectable in the control cell lysates (Fig. 3A), Rac1 immunoreactivity was very weak, although equal amounts of total protein was used for these analyses (Fig. 3B). This difference suggests that Rac1 may predominantly localize to the lens cell membrane. These results depicted in Figure 3 demonstrate that lovastatin treatment leads to the accumulation of RhoA and Rac1 in the soluble fraction of lens epithelial cells compared with control cell lysates. These data suggest that lovastatin-mediated reduction in isoprenoid synthesis impairs isoprenylation-dependent membrane localization of RhoA and Rac1 GTPases.

Reversal of Lovastatin-Induced Changes by Isoprenoid Supplementation

To determine the identity of the small GTPases, whose isoprenylation is the most likely function affected by lovastatin in lens epithelial cells, we examined the ability of isoprenoid supplementation to reverse the effects of lovastatin on both human and porcine lens epithelial cells. Addition of geranylgeranyl or farnesyl pyrophosphate (10 \( \mu M \)) to the medium of lovastatin-treated (20 \( \mu M \) for 18 hours; Fig. 4) cells revealed that only geranylgeranyl pyrophosphate possessed the ability to support the dramatic recovery of cell morphology to the normal state (Fig. 4). The reversal of cell shape changes induced by lovastatin was very rapid and occurred within 4 hours of geranylgeranyl supplementation of human lens cells (Fig. 4C). Porcine primary cultures required an 18- to 24-hour period for recovery of morphologic changes. In contrast, supplementation with farnesyl pyrophosphate for 24 hours had a marginal effect on cell morphology in both cell types (Fig. 4D).

In addition to the ability of isoprenoid supplementation to reverse cell morphology, its ability to restore cytoskeletal changes was also addressed by staining lovastatin-treated, isoprenoid-supplemented lens epithelial cells for actin, vinculin, and phosphotyrosine. Figure 5 illustrates the recovery of actin stress fibers, focal adhesions, and phosphotyrosine. Arrows: specific staining of focal adhesions and phosphotyrosine.
adhesions and tyrosine phosphorylated proteins in these cells. Both human and porcine cells showed similar recovery of actin stress fibers and focal adhesions in response to geranylgeranyl pyrophosphate supplementation. Control cells showed no obvious effects, either on cell morphology or cytoskeletal reorganization, in response to addition of geranylgeranyl or farnesyl isoprenoids.

Supplementation with geranylgeranyl pyrophosphate also reversed the lovastatin-induced accumulation of RhoA and Rac1 in the cytosolic fraction of lens epithelial cells; resulting in distribution profiles characteristic of untreated control cells (Fig. 3).

C3-Exoenzyme–Induced Changes in Cell Shape and Actin Cytoskeleton

To selectively identify Rho GTPase-induced effects in lovastatin-treated cells, Rho GTPase was irreversibly inactivated with C3-exoenzyme.\textsuperscript{1,2} by pretreating the cells with C3-exoenzyme before the addition of lovastatin and subsequent supplementation with geranylgeranyl pyrophosphate, as described earlier. C3 treatment alone led to cell shape changes and loss of actin stress fibers in human lens cells (Fig. 6C). The actin staining of cells treated with C3, lovastatin, and geranylgeranyl pyrophosphate (Fig. 6G) was compared with that of cells treated with lovastatin and geranylgeranyl pyrophosphate (Fig. 6E). Compared with lovastatin- and geranylgeranyl-treated cells (Fig. 6E), in which most of the actin stress fibers and cell shape reverted to normal, cells treated with C3, lovastatin, and geranylgeranyl pyrophosphate (Fig. 6G) had markedly reduced actin stress fibers with altered cell shape. Geranylgeranyl supplementation of C3-exoenzyme–treated cells did not cause significant recovery of actin stress fibers and cell shape (Fig. 6F). The C3-mediated changes and the inability to recover actin stress fibers and cell shape in lens cells treated with C3 before lovastatin exposure and supplementation with geranylgeranyl pyrophosphate clearly indicate the specific involvement of Rho GTPases in lovastatin-induced changes.

DISCUSSION

In the present study, lovastatin induced disassembly of the actin cytoskeleton and loss of cell adhesions in lens epithelial cells, and the impairment of geranylgeranylation of the target protein(s) is thought to be responsible for these effects. The critical role of protein geranylation in the maintenance of cell shape and cytoskeletal organization and in cell–cell and cell–extracellular matrix adhesive function was demonstrated by the ability of geranylgeranyl pyrophosphate supplementation, to specifically reverse the effects of lovastatin in lens epithelial cells.

Rho GTPases (including Rho, Rac, and Cdc42) participate in regulating actin cytoskeletal organization and various aspects of cell adhesion properties. Rho regulates the formation of actin stress fibers and focal adhesions, cell morphology, and smooth muscle contraction,\textsuperscript{1,2} and Rac regulates membrane ruffling, actin polymerization, and cadherin-mediated cell–cell adhesions.\textsuperscript{3} Cdc42 mediates formation of filopodia and intercellular adhesive interactions.\textsuperscript{1–3} Each of these GTPases is also involved in the regulation of gene transcription and cell survival.\textsuperscript{4} Rho, Rac, and Ras GTPases are well-characterized pro-
teins whose functions depend on posttranslational isoprenylation catalyzed by farnesyl transferase (Ras GTase) and geranylgeranyl transferases (Rho and Rac GTases).\(^7\)^\(^8\) Lovastatin impairs both types of isoprenylation by inhibiting the synthesis of mevalonic acid, which is a precursor of the isoprenoids farnesyl and geranylgeranyl pyrophosphates.\(^7\)^\(^8\)

In lens epithelial cells, however, lovastatin-induced changes in cell shape, actin cytoskeletal organization, and protein tyrosine phosphorylation were completely reversed by supplementation with geranylgeranyl pyrophosphate, whereas farnesyl pyrophosphate had no effect (Fig. 5). Although the level of protein tyrosine phosphorylation seemed to increase in lovastatin-treated cells after supplementation with farnesyl pyrophosphate (Fig. 5), lovastatin-induced alterations in cell morphology, actin stress fiber formation, and cell adhesion were not reversed. Rho and Rac are geranylgeranylated GTases, and this isoprenylation is critical for membrane localization and thus for the function of these proteins.\(^7\) Accumulation of RhoA and Rac1 in the soluble fractions of lovastatin-treated lens epithelial cells and the reversal of this effect by geranylgeranyl pyrophosphate supplementation (Fig. 3) convincingly demonstrated that lovastatin treatment indeed impaired the isoprenylation and function of RhoA and Rac1. Additionally, the inability of geranylgeranyl pyrophosphate supplementation to reverse lovastatin-induced changes in actin and cell shape in lens epithelial cells pretreated with the Rho GTase-specific inhibitor C3-exoenzyme demonstrated the specific role of Rho GTase in actin cytoskeletal organization and cell morphology (Fig. 6). Data from isoprenoid supplementation and C3 experiments, when taken together with lovastatin-induced alterations in RhoA and Rac1 distribution in lens cells, strongly suggest an involvement of geranylgeranylated GTases such as Rho and Rac in lovastatin’s effects.

Rho and Rac GTases also play an important role in regulating cell death through cytoskeletal organization and cell–cell and cell–ECM interactions.\(^2\)^\(^3\)^\(^19\)^\(^20\) Cell–cell and cell–ECM adhesions are crucial for the growth and survival of multicellular organisms.\(^22\)^\(^25\) Loss of cell adhesions (both focal and cadherin-mediated adhesions) has been shown to lead to cell death through increased apoptosis.\(^22\)^\(^25\) Therefore, impaired function of Rho and Rac GTases in lovastatin-treated lens epithelial cells could increase apoptotic stress by affecting the integrity of focal and cell–cell adhesions. Both cell–cell and cell–ECM junctional complexes are rich in phosphotyrosinylated proteins, which are central to regulating cell–cell and focal adhesions.\(^1\)^\(^3\)^\(^22\)^\(^25\) Lovastatin markedly decreased focal adhesions, cell–cell adhesions, and protein phosphotyrosine in lens epithelial cells (Figs. 1, 2). Lens epithelial cells treated with lovastatin also showed increased caspase-3 activity and gelsolin fragmentation, suggesting increased apoptotic stress.\(^26\)^\(^29\) Associated with impaired activity of the small GTases (Maddala et al., unpublished data, 2001).

We have reported previously the distribution of small GTases, localized predominantly to the membrane fractions of lens tissue,\(^4\) suggesting that most of the GTases are isoprenylated. Our studies have also revealed that mevalonic acid (the precursor of isoprenoids) supplementation prevents lovastatin-induced cataract in organ-cultured rat lenses.\(^5\) Furthermore, supplementation with geranylgeranyl, but not farnesyl pyrophosphate, prevents cataractogenesis induced by lovastatin in rat lenses (manuscript under preparation, 2001, Sam Zigler, National Eye Institute, Bethesda, MD). Based on these observations and together with the data presented in this study, it is reasonable to infer that Rho- and Rac-regulated cytoskeletal organization is potentially important in the maintenance of lens transparency and function.

In conclusion, the results presented in this study confirm that treatment of lens epithelial cells with lovastatin impairs the function of geranylgeranylated small GTases, such as Rho and Rac GTases, by inhibiting their isoprenylation. This effect ofLovastatin on GTases leads to disassembly of actin stress fibers, impaired protein tyrosine phosphorylation, and loss of focal and cell–cell adhesive interactions. These cytoskeletal changes lead to altered cell morphology and potentially to cell death. 

**Figure 5.** Effect of isoprenoid supplementation on lovastatin-induced cytoskeletal changes in human lens epithelial cells. Confluent human lens epithelial cells grown on gelatin-coated glass coverslips were treated with 20 \(\mu M\) lovastatin for 18 hours and subsequently supplemented with either 10 \(\mu M\) geranylgeranyl pyrophosphate (GGPP) or farnesyl pyrophosphate (FPP). Lovastatin-treated and isoprenoid-supplemented cells were stained for actin stress fibers, focal adhesions, and phosphotyrosine (Py). Lovastatin-induced actin depolymerization, loss of focal adhesions, and phosphotyrosine were reversed back to normal after supplementation with geranylgeranyl alone, but not after supplementation with farnesyl pyrophosphate. Similar results were observed with porcine lens epithelial cells (data not shown). The results observed with combined supplementation of geranylgeranyl and farnesyl pyrophosphates were found to be very similar to those observed with geranylgeranyl pyrophosphate alone (data not shown).
death through increased apoptosis. Thus, synthesis of meva-
lonic acid and generation of isoprenoid precursors affect
the function of isoprenylated small GTPases that are critical for
lens epithelial cell morphology and survival.

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