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Effects of lovastatin on Rho isoform expression, activity, and association with guanine nucleotide dissociation inhibitors

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ABSTRACT

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC1.1.1.88) inhibitors (statins) reduce cholesterol synthesis and prevent cardiovascular disease; they can also inhibit prenylation of Ras and Rho proteins, and have anti-neoplastic effects. Rho proteins cycle between an active, GTP-bound, and an inactive, GDP-bound form, and Rho prenylation is important for Rho's interaction with upstream regulators and downstream effectors, but the effects of statins on Rho signaling are incompletely understood. We found that the HMG-CoA reductase inhibitor lovastatin markedly induced the expression of RhoA, B, and C in human erythroleukemia (HEL) cells. The drug increased RhoA and C only in their unprenylated forms, but it increased both prenylated and unprenylated RhoB and did not significantly affect N- and K-Ras prenylation, suggesting that it inhibited geranyl-geranylation more efficiently than farnesylation. Quantitative analysis of nucleotides bound to Rho demonstrated a 3.7-fold increase in Rho-GTP and a similar increase in Rho-GDP in lovastatin-treated cells, leaving the fraction of Rho in the active, GTP-bound form constant at 5.8%. Lovastatin reduced Rho association with Rho guanine dissociation inhibitor (RhoGDI)-α and -β, and prenylation-deficient Rho mutants did not associate with RhoGDI. siRNA inhibition of RhoGDIα expression increased Rho-GTP, suggesting that decreased Rho/RhoGDIα association explained an increase in unprenylated Rho-GTP in lovastatin-treated cells. Unprenylated Rho A, B, and C were partly functional in activating serum response element-dependent transcription. In conclusion, we quantified effects of lovastatin on RhoA, B, and C isoforms, and provide a molecular mechanism whereby statins cause accumulation of unprenylated Rho-GTP.

1. Introduction

Rho GTPases (Rho, Rac, and CDC42) regulate cell growth and apoptosis, and cytoskeletal dynamics involved in cell motility, invasion, and metastasis [1]. Rho proteins cycle between an inactive, GDP-bound and an activated, GTP-bound state; they are activated by guanine nucleotide exchange factors (GEFs), and inactivated by GTPase-activating proteins (GAPs). Rho-

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Abbreviations: GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GST, glutathione-S-transferase; HEL cells, human erythroleukemia cells; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; RBD, Rho binding domain.

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GDP is mainly cytoplasmic and associated with GDP dissociation inhibitors (GDIs), whereas Rho-GTP is thought to interact with effector proteins at the plasma membrane [2]. Three highly related (85% amino acid identity) Rho isoforms, RhoA, B, and C, appear to interact with the same GEFs and share some of the same effector proteins, but show clear functional differences. In fibroblasts and cancer cells, RhoA and C are predominantly *pro*-proliferative and *anti*-apoptotic, while RhoB is *anti*-proliferative and *pro*-apoptotic [1]. RhoA is required for Ras-induced cellular transformation, and shows increased activation in Ras-transformed cells [1,3]. Activating Rho mutations have not been reported in human malignancies, but several Rho-regulatory proteins were found in screens for transforming genes, and in leukemia-associated chromosomal translocations [1]. In various human cancers, RhoA or C overexpression is associated with a more aggressive phenotype [1].

Rho proteins are post-translationally modified by an isoprenoid group—RhoA and C exclusively by a geranyl-geranyl group, but RhoB by either a geranyl-geranyl or a farnesyl group [4]. Prenylation of Rho proteins is essential for proper membrane localization, and appears to be required for most, but not all, known Rho functions [5–8]. HMG-CoA reductase (EC1.1.1.88) inhibitors (statins) reduce the synthesis of mevalonate, the precursor of cholesterol, and of farnesyl and geranyl-geranyl isoprenoids [9]. In addition to lowering serum cholesterol levels, statins exert ''pleiotropic'' effects such as reducing vascular inflammation, and improving endothelial function [10]. At clinically achievable concentrations, statins also induce growth arrest and apoptosis in leukemic and other malignant cells, and promising anti-tumor activities have been observed in animal models and early clinical trials [9,11]. It has been proposed that many of these pleiotropic effects are mediated by statins inhibiting Ras and Rho prenylation [10].

Since GEFs activate prenylated Rho in vitro much more efficiently than unprenylated Rho, statins are generally assumed to block Rho activation [5]. However, in vitro studies suggest that the interaction of Rho with GAPs and GDIs may also be affected by Rho's prenylation state, and in vitro studies may not predict the effect of prenylation inhibitors on Rho activity in intact cells. There are conflicting reports in the literature, whether statins increase or decrease Rho-GTP levels in intact cells [12–16]. Therefore, we decided to study the effects of lovastatin on Rho signaling in human erythroleukemia (HEL) cells. We found that lovastatin caused accumulation of unprenylated RhoA, B, and C in the GTP-bound form, and determined the molecular mechanism of the increase in Rho-GTP.

2. Materials and methods

2.1. Materials and DNA constructs

Antibodies directed against the following proteins were from Santa Cruz Biotechnology: RhoA (sc-418), RhoB (sc-180), RhoC (sc-26480), H-Ras (sc-29), K-Ras (sc-30), actin (sc-8432), RhoGDIα (sc-360), and RhoGDIβ (sc-11359). Rho standards were purified from bacteria to >90% homogeneity as judged by PAGE/Coomassie staining, and were quantified by comparison to bovine serum albumin. Glutathione-S-transferase(GST)-tagged rhotekin Rho binding domain (RBD) was purified from bacteria as described previously [3,17]. Vectors encoding RhoA and B with the N-terminal epitope tag EEEEYMPME (EE-tag) were constructed as described [18]; vectors encoding hemagglutinin (HA)-tagged RhoC (wild type and V14) were purchased from the University of Misouri-Rolla cDNA Resource Center. Prenylation-deficient mutants of RhoA (S190), RhoB (S193), and RhoC (S190) were obtained by PCR using antisense primers with the appropriate nucleotide changes; all PCR products were sequenced. Lovastatin lactone (Calbiochem) was converted to the active acid as described [19].

2.2. Cell culture

HEL cells were cultured in RPMI supplemented with 10% fetal bovine serum. For most experiments, mid-log phase cells were diluted to a density of 2×10^5 cells/ml and exposed for 48 h to 10 μM lovastatin, unless stated otherwise.

2.3. Measurement of Rho activation

Two different methods were used to measure Rho activation; both rely on the Rho binding domain (RBD) of rhotekin to isolate Rho-GTP. To determine the activation state of each Rho isoform separately, we performed the assay as described by Ren et al. [17], assessing the amount of GTP-bound RhoA, B, or C by Western blotting. To quantify absolute amounts of GTP and GTP + GDP bound to Rho, we employed a coupled enzymatic assay using nucleoside diphosphate kinase and luciferase as described previously [3,18].

2.4. Triton X-114 phase separation

Rho proteins were analyzed for isoprenylation by Triton X-114 partition [20]. Cells were lysed in ice-cold buffer containing 1% Triton X-114, and after a 2 min centrifugation, the supernatant was warmed to 30 °C for 1 min, and micelles formed were pelleted by centrifugation at room temperature. The aqueous supernatant was cooled to 4 °C, and adjusted to 1% Triton X-114; the detergent pellet was diluted to the same volume as the aqueous phase using lysis buffer without detergent. Equal volumes of the two fractions were analyzed by SDS-PAGE/Western blotting [18], or were incubated with GST-rhotekin-RBD for measuring absolute amounts of GTP and GDP + GTP as described above [3].

2.5. DNA transfection, co-immunoprecipitation, and reporter gene assays

HEL cells were transfected using either the Nucleofector™ (Amaxa) protocol (program X-005, solution V), or using DMRIE-C™ transfection reagent (InVitrogen). Human embryonal kidney (293T) cells were transfected by calcium phosphate precipitation. Cells were lysed by Dounce-homo-genization, centrifuged, and supernatants were subjected to immunoprecipitation. Luciferase and β-galactosidase reporter gene activities were measured as described previously [18].

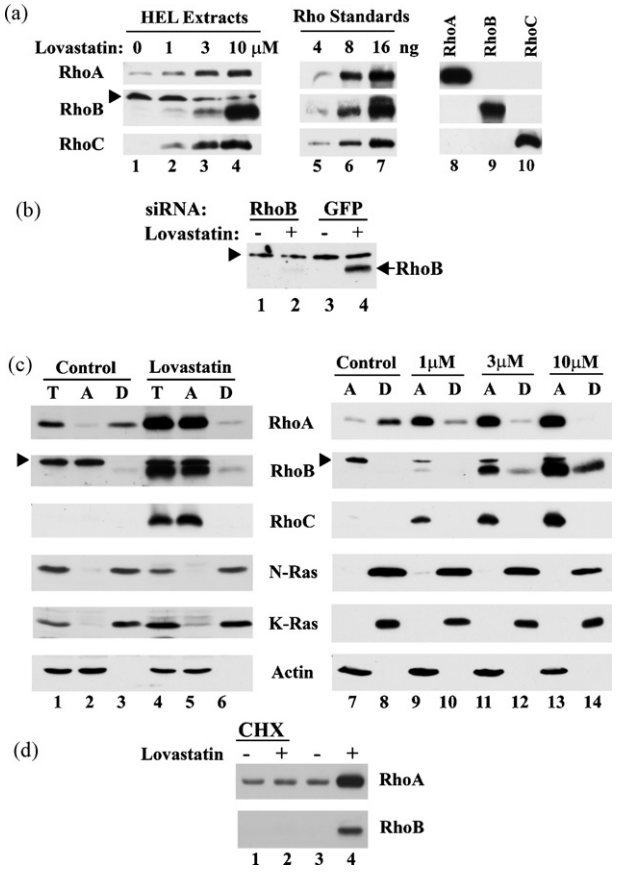

Fig. 1 – Lovastatin increases expression of unprenylated RhoA, B, and C in HEL cells. Panel (a): HEL cells were treated for 48 h with increasing concentrations of lovastatin (lanes 1–4); 5, 150, or 100 μg (for RhoA, B, or C, respectively) of extracted cellular protein was analyzed by SDS-PAGE/Western blotting using antibodies specific for each Rho isoform. Purified RhoA, B, or C (4, 8, and 16 ng) were analyzed in parallel (lanes 5–7). To determine antibody specificity, 40 ng of purified Rho A, B, or C were loaded in lanes 8–10 as indicated. The arrowhead on this and subsequent RhoB blots denotes a protein of slightly higher molecular weight than RhoB that cross-reacts with the RhoB antibody. Panel (b): HEL cells were transfected with siRNA oligoribonucleotides specific for RhoB (lanes 1 and 2) or with control siRNA specific for GFP (lanes 3 and 4), and were cultured in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 3 μM lovastatin for 48 h. Cell lysates were analyzed by Western blotting using the RhoB-specific antibody described in panel (a). Panel (c): HEL cells were cultured for 48 h in the absence (control, lanes 1–3 and 7–8) or presence of lovastatin (10 μM lovastatin in lanes 4–6; lovastatin concentration as indicated in lanes 9–14). Cells were extracted in 1% Triton-X-114, and total cell lysates (T) were separated into an aqueous supernatant (A), and a detergent-containing pellet (D). Aliquots of total cell lysate, aqueous supernatant (containing unprenylated proteins), and detergent pellet (containing prenylated proteins) were analyzed by SDS-PAGE/Western blotting. The actin blot demonstrated minimal protein loss during extraction, and no contamination of the detergent pellet with proteins from the aqueous phase. Panel (d): HEL cells were pre-treated with 2 μg/ml of cycloheximide (CHX, lanes 1 and 2) or left untreated (lanes 3 and 4) prior to the addition of 10 μM lovastatin for 24 h (lanes 2 and 4). Whole cell lysates were analyzed by Western blotting as described in panel (a).

2.6. siRNA transfection experiments

The siRNAs for green fluorescent protein (GFP) was described previously [21]. To silence RhoB expression, we employed Smartpool siRNA reagent M-008395-0005 from Dharmacon. The siRNA for RhoGDIα was directed against the target sequence 5′-CAGGAAAGGCGUCAAGAUUGA-3′ [22]. siRNAs were introduced into HEL cells using the Nucleofector™ protocol (Amaxa, program T-016, solution V).

2.7. Data analysis

Bar graphs represent the mean ± S.D. of at least three independent experiments; Western blots represent typical experiments reproduced at least three times with similar results. We employed the Student's *t*-test for comparison of two groups, and ANOVA for multiple comparisons; differences were considered significant at $p < 0.05$.

3. Results

3.1. Lovastatin increases expression of unprenylated RhoA, B, and C in HEL cells

Treating HEL cells for 48 h with increasing concentrations of lovastatin from 1 to 10 μM progressively increased the amount of RhoA, B, and C (Fig. 1a, lanes 1–4). RhoA and B antibodies were about equally sensitive in detecting their respective proteins, and more sensitive than the RhoC antibody (Fig. 1a, lanes 5–7; antibody specificity is shown in lanes 8–10. Please note that 5, 150, or 100 μg of cellular protein was loaded per lane to generate the blots for RhoA, B, and C, respectively). Taking into consideration the amounts of cell protein used to generate the blots in panel (a), we estimate that RhoA constitutes >95% of the three Rho proteins in untreated HEL cells, and that it increased several-fold in cells treated with 10 μM lovastatin. RhoB and C were below the limits of detection in untreated cells, but constituted about 5 and 10% of Rho proteins, respectively, in lovastatin-treated cells. The band marked with an arrowhead on the RhoB blot represents a cross-reacting protein that migrates with a slightly higher molecular mass than RhoB. To further demonstrate specificity of the RhoB antibody, we treated HEL cells with siRNA oligoribonucleotides targeting RhoB mRNA to prevent RhoB induction by lovastatin. As shown in Fig. 1b, the band representing RhoB was induced by lovastatin in cells treated with a control siRNA targeted at green fluorescent protein (GFP), but it was abolished in RhoB siRNA-treated cells; in contrast, the non-specific band (marked again with an arrowhead) was not affected by the RhoB siRNA.

To quantify Rho prenylation, we extracted cells in the low cloud-point detergent Triton X-114. This allows separating

extracts into an aqueous and detergent phase, and previous work showed that unprenylated Ras and Rho proteins partition into the aqueous phase, while prenylated proteins partition into the detergent phase [7,20]. In the absence of lovastatin, RhoA was largely in the detergent phase (Fig. 1c), and RhoB and C were undetectable (the cross-reacting protein recognized by the RhoB antibody is again marked with an arrowhead and partitioned in the aqueous phase). Treating HEL cells for 48 h with 10 μM lovastatin caused almost all RhoA, B, and C to partition into the aqueous phase, indicating nearly complete inhibition of Rho prenylation (Fig. 1c, left half of panel). The phase separation appeared efficient without loss of cellular proteins, because similar amounts of N- and K-Ras, which are farnesylated, were found in the detergent phase and total cell lysate, and similar amounts of α-actin, which is not prenylated, were in the aqueous phase and lysate (Fig. 1c, lower three panels). From 1 to 10 μM lovastatin, we found a progressive shift of RhoA into the aqueous phase, and RhoC appeared exclusively in the aqueous phase (Fig. 1c, right half of panel). RhoB appeared predominantly in the aqueous phase, but a significant amount of RhoB was found in the detergent phase, which may represent farnesylated RhoB (Fig. 1c; see Section 4). Lovastatin did not shift N- and K-Ras into the aqueous phase, suggesting that protein farnesylation was less affected than protein geranyl-geranylation up to 10 μM lovastatin.

To determine whether the accumulation of Rho proteins in lovastatin-treated HEL cells required de novo protein synthesis, we pre-incubated cells for 1 h with the protein synthesis inhibitor cycloheximide prior to the addition of lovastatin. Cycloheximide completely prevented the lovastatin-induced increase in Rho protein, indicating that the accumulation of Rho in lovastatin-treated cells was due to new protein synthesis (Fig. 1d shows results for RhoA and B).

Thus, lovastatin markedly increased the amount of unprenylated RhoA, B, and C in HEL cells, without affecting expression or prenylation of N- and K-Ras. Similar results were obtained with mevastatin (data not shown).

3.2. Lovastatin increases the amount of GTP-bound RhoA, B, and C in HEL cells

Because prenylation is required for Rho proteins to interact with both their activators and inhibitors [5,23,24], an increase in unprenylated RhoA, B, and C could either increase or decrease the amount of the proteins in the activated GTP-bound state. Using a Rhotekin pull-down assay of GTP-bound Rho proteins, we found a striking increase in the amount of activated, GTP-bound RhoA, B, and C in HEL cells treated with 10 μM lovastatin (Fig. 2, lanes 2–5).

To determine if lovastatin increased the percent of Rho proteins in the activated GTP-bound state [i.e., Rho-GTP/(Rho-GTP + Rho-GDP) × 100], we used a quantitative assay that measures Rho-GTP, and the sum of Rho-GTP plus Rho-GDP [18]. We found a 3.7-fold increase in the absolute amount of Rho-GTP (Fig. 3a, inset), but the percent activation of Rho proteins remained constant at 5.8% after lovastatin treatment, indicating a proportional increase in both Rho-GTP and Rho-GDP (Fig. 3a). This method does not distinguish among GTP-bound RhoA, B, or C, but the measured increase in GTP-bound

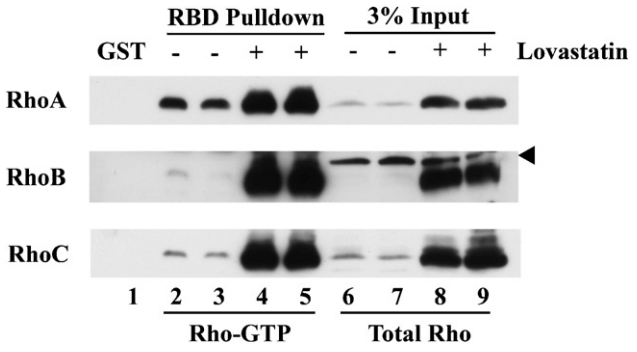

Fig. 2 – Lovastatin increases the amount of GTP-bound RhoA, B, and C in HEL Cells. HEL cells were cultured for 48 h in the absence (lanes 2, 3, 6, and 7) or presence (lanes 4, 5, 8, and 9) of 10 μM lovastatin. Cell lysates were incubated with a rhotekin-RBD-GST-fusion protein bound to glutathione agarose beads to isolate GTP-bound Rho proteins. Beads coated with GST alone served as a control (GST, lane 1). GTP-bound RhoA, B, or C associated with the rhotekin-RBD (RBD pulldown, lanes 2–5) and total RhoA, B, or C present in 3% of the whole cell lysate (input, lanes 6–9) were assessed by Western blotting. As described in the legend of Fig. 1, the arrowhead denotes a cross-reacting protein detected by the RhoB antibody.

Rho occurred largely on RhoA, because RhoA constituted >95% of Rho proteins in untreated and >85% in lovastatin-treated cells.

Since prenylation affects the interaction of Rho with its regulatory proteins in vitro, the activation states of prenylated and unprenylated Rho could be different. To address this question, we analyzed Rho isolated from the aqueous and detergent phases of Triton X-114-extracted cells, realizing that the absolute amounts of nucleotide-bound Rho in the aqueous and detergent phase cannot be directly compared (because the two phases have different protein concentrations). However, the percent activation can be compared, and, in untreated cells, we found a trend towards higher Rho activation in Rho isolated from the detergent phase than from the aqueous phase: 9.5 ± 3.2% compared to 5.3 ± 1.1%, respectively (Fig. 3b). In lovastatin-treated cells, Rho activation in the aqueous phase was 6.1 ± 1.5% (Fig. 3b, left panel), which is similar to that of Rho isolated from whole cell extracts. We could not determine the level of Rho activation in the detergent phase of lovastatin-treated cells, because of the extremely small amount of prenylated Rho in this condition.

In conclusion, lovastatin increased the amount of activated, GTP-bound RhoA, B, and C, but the proteins were unprenylated. Similar results were obtained with mevastatin (data not shown). The amount of GDP-bound Rho, at least for RhoA, increased proportionately with the amount of GTP-bound Rho, leaving the percent activation unchanged.

3.3. Lovastatin decreases Rho association with RhoGDI

Under basal conditions, GDP-bound Rho proteins are thought to be associated primarily with RhoGDIs, and, after cellular

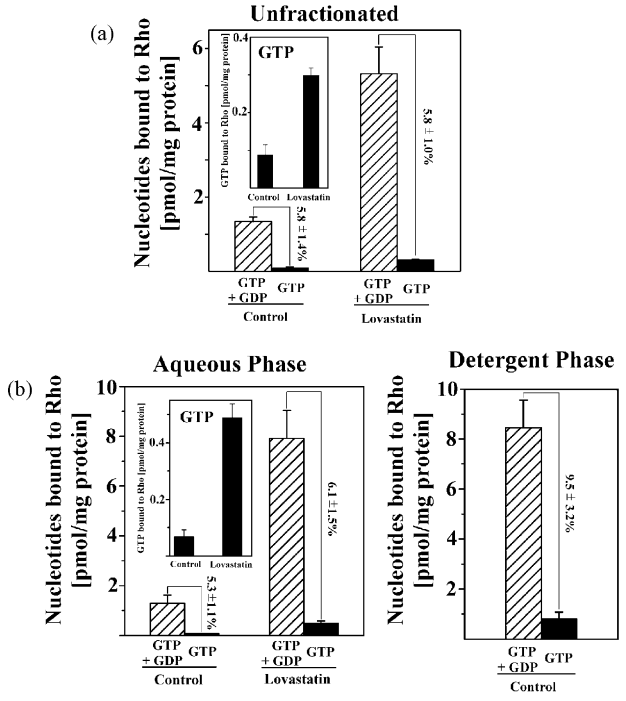

Fig. 3 – Quantification of GTP and GDP bound to prenylated vs. unprenylated Rho. Panel (a): HEL cells were cultured and extracted as described in Fig. 2, and the amounts of total nucleotides (GTP + GDP, striped bars) or GTP (filled bars) bound to Rho in unfractionated cell lysates were measured enzymatically. The percent of Rho in the active, GTP-bound form is indicated. The inset shows GTP-bound Rho on an expanded scale. Panel (b): HEL cells were cultured in the absence or presence of 10 μM lovastatin and extracted in Triton-X-114. Unprenylated Rho proteins present in the aqueous phase (left panel) and prenylated Rho proteins present in the detergent phase (right panel) were subjected to an RBD pulldown assay; the amounts of total nucleotides (GTP + GDP, striped bars) or GTP (filled bars) bound to Rho were measured enzymatically. The inset in the left panel shows GTP-bound Rho on an expanded scale. The amount of Rho present in the detergent phase in lovastatin-treated cells was below the limit of detection. The detergent phase had a lower protein concentration than the aqueous phase, making the denominator for the amount of nucleotides bound to Rho different between the two phases. In panels (a and b), the comparison between control and lovastatin-treated cells showed a significant difference for both total nucleotides (GTP + GDP) and GTP bound to Rho ($p < 0.05$).

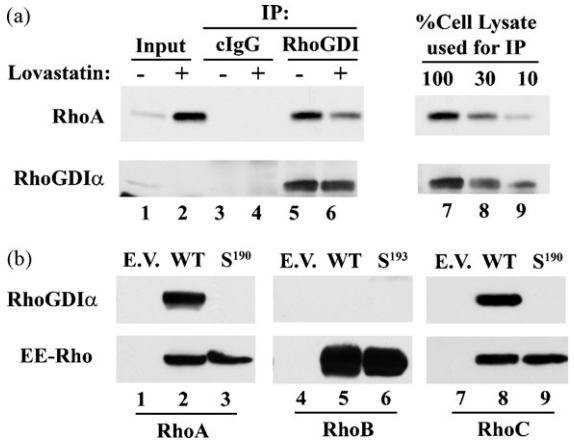

Fig. 4 – Lovastatin decreases RhoA association with RhoGDI. Panel (a): HEL cells were cultured for 48 h in the absence (lanes 1, 3, 5, and 7–9) or presence of 10 μM lovastatin (lanes 2, 4, and 6), and cell extracts were subjected to immunoprecipitation with a RhoGDIα-specific antibody (lanes 5–9) or control IgG (lanes 3 and 4). Immunoprecipitates were analyzed by Western blotting with an antibody specific for RhoA (upper panels); blots were stripped and reprobed with an antibody for RhoGDIα (lower panels). Input cell lysates were analyzed in parallel (lanes 1 and 2; 3% of total lysate). The amount of RhoA co-immunoprecipitated with RhoGDIα was proportional to the amount of RhoGDI in the immunoprecipitates as demonstrated in the right half of the panel: lanes 7, 8, and 9 show 100, 30, and 10% of the cell lysate, respectively, subjected to immunoprecipitation. Panel (b): Human embryonal kidney (293T) cells were transfected with empty vector (E.V., lanes 1, 4, and 7), epitope-tagged wild type Rho (WT, lane 2 for RhoA, lane 5 for RhoB, and lane 8 for RhoC) or prenylation-deficient, mutant Rho constructs (lane 3 for $RhoA^{S190}$, lane 6 for $RhoB^{S193}$, and lane 9 for $RhoC^{S190}$). Cell lysates were subjected to immunoprecipitation using an epitope-specific antibody, and precipitates were analyzed by Western blotting using a RhoGDIα antibody (upper panel). Blots were stripped and re-probed with antibodies for RhoA (lanes 1–3), RhoB (lanes 4–6), or RhoC (lanes 7–9).

stimulation, they dissociate from the RhoGDI, and are activated by a GEF-mediated exchange of GTP for GDP [2]. To study the mechanism of the lovastatin-induced increase in Rho-GTP, we assessed Rho/RhoGDIα association, and found about 70% less RhoA associated with RhoGDIα in lovastatin-treated HEL cells compared to untreated cells (Fig. 4a, compare lanes 5 and 6). This finding was particularly striking considering the large increase in Rho-GDP (Fig. 3a) and total RhoA (Fig. 4a, lane 2) that occurred in cells treated with lovastatin. Co-immunoprecipitation of RhoA and RhoGDIα increased proportionally with increasing amounts of cell extract, indicating quantitative recovery within the limits of the assay (Fig. 4a, lanes 7–9). Thus, as observed in vitro with purified proteins [24], unprenylated Rho showed decreased association with RhoGDIα in vivo, and the decreased association could explain the increase in Rho-GTP.

The interaction between RhoGDI and Rho is mediated by lipid–protein and protein–protein interactions, and regulated by proteins competing with RhoGDI for Rho binding [2]. To examine the association of RhoB and C with RhoGDIα, and to determine if the prenylation state of Rho alone explains the decreased Rho/RhoGDI association observed in lovastatin-treated cells, we transfected 293T cells with epitope-tagged wild type and prenylation-deficient Rho constructs. The prenylation-deficient Rho proteins have a serine replacing

the C-terminal cysteine in the CAAX box, which prevents prenylation as well as subsequent post-translational modifications [4]. When wild type and mutant RhoA^{S190} or RhoC^{S190} were isolated by immunoprecipitation, RhoGDIα was present in the immunoprecipitates of the wild type, but not the non-prenylated Rho proteins (Fig. 4b). No interaction of RhoGDIα with either wild type or non-prenylated RhoB could be detected, although large amounts of RhoB were present in the immunoprecipitates. This finding is consistent with previous work showing that RhoA, but not RhoB, interacts with fluorescently-labeled RhoGDIα in living cells [25].

To determine if RhoB associated with RhoGDIβ (LyGDI), a hematopoietic cell-specific GDI that binds RhoA, Rac, and CDC42, albeit less efficiently than RhoGDIα [26], we transfected HEL cells with epitope-tagged Rho constructs, and examined anti-epitope immunoprecipitates for RhoGDIβ. Small amounts of RhoGDIβ were associated with wild type, but not unprenylated RhoA; no RhoGDIβ was detectable in the immunoprecipitates containing wild type or unprenylated RhoB (data not shown).

Thus, only prenylated, but not unprenylated RhoA and RhoC associated with RhoGDIα (and RhoGDIβ for RhoA) in intact cells. RhoB may be associated with RhoGDIγ, a recently described GDI which is found in a detergent-insoluble cytoskeletal fraction and interacts specifically with post-translationally modified RhoB in vitro [27]. The lack of available RhoGDIγ antibodies and poor protein solubility precluded us from studying this interaction in intact cells.

3.4. *RhoGDIα knock-down increases the amount of Rho-GTP*

To determine whether changing Rho association with RhoGDIα is sufficient to change Rho GTP-loading, we used an siRNA strategy to reduce RhoGDIα expression. Transfecting HEL cells with a RhoGDIα-specific siRNA substantially reduced RhoGDIα compared to cells transfected with a control siRNA targeted against GFP (Fig. 5a, top panel). The RhoGDIα siRNA reduced RhoA, whereas it had no effect on K-Ras, Rap1A, or tubulin (Fig. 5a; tubulin not shown). These findings suggest that lack of RhoGDIα binding to prenylated RhoA may render RhoA unstable, similar to Rac1 and CDC42, which are destabilized in RhoGDIα-deficient cells due to increased proteolysis [22]. In contrast, *un*prenylated RhoA is less susceptible to proteolysis than prenylated RhoA [28], which can explain its accumulation in lovastatin-treated cells, despite decreased association with RhoGDIα.

To determine the effect of decreased Rho/RhoGDIα association on Rho-GTP levels, we measured GTP and GTP + GDP bound to Rho in HEL cells transfected with RhoGDIα siRNA, or control siRNA. Total GTP + GDP bound to Rho decreased by about 20% in cells transfected with the RhoGDIα-specific siRNA, compared to control siRNA-transfected cells (Fig. 5b); this agrees with the decreased RhoA observed on Western blots (Fig. 5a). The amount of Rho-GTP increased significantly in the RhoGDIα-deficient cells, increasing the percent of Rho in the activated, GTP-bound state from 3.9 ± 0.6 to 8.3 ± 1.1% in control versus RhoGDIα siRNA-transfected cells, respectively (Fig. 5b). Thus, decreased association between Rho and RhoGDIα leads to increased Rho-GTP levels. These results also suggest that RhoGDIα is a major RhoA-binding GDI in HEL cells.

3.5. *Unprenylated RhoA, B, and C exhibit partial transcriptional activity*

Rho activation of a serum response factor/megakaryoblastic leukemia 1 (MKL1) complex is required for transcriptional regulation of genes controlled by serum response elements (SREs); a *t*(1:22) translocation in acute megakaryoblastic

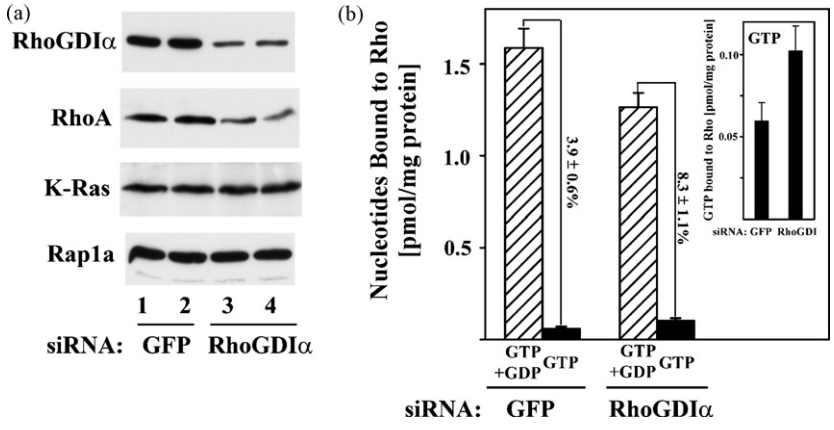

Fig. 5 – Decreased Rho/Rho-GDI binding causes increased Rho-GTP. Panel (a): HEL cells were transfected with siRNA oligoribonucleotides specific for RhoGDIα (lanes 3 and 4) or with control siRNA specific for GFP (lanes 1 and 2). Cells were extracted 48 h later, and the extracts were subjected to SDS-PAGE/Western blotting using antibodies specific for RhoGDIα (top panel), RhoA (second panel), K-Ras (third panel), and Rap1A (lower panel). Panel (b): HEL cells were transfected as described in panel (c); after 48 h, cells were extracted and total nucleotides (GTP + GDP, striped bars) or GTP (filled bars) bound to Rho were measured as described in the legend to Fig. 3a. The percent of total Rho in the active, GTP-bound state is indicated for each condition, and the inset shows GTP-bound Rho on an expanded scale. Comparing cells treated with control vs. RhoGDI-specific siRNA yielded a significant difference in both total nucleotides (GTP + GDP) and GTP bound to Rho ($p < 0.05$).

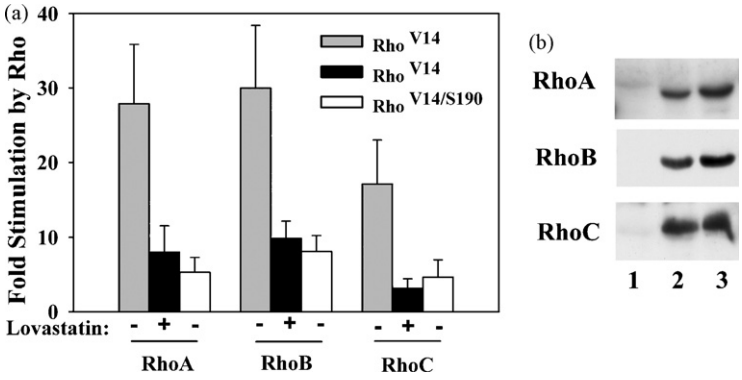

Fig. 6 – Unprenylated RhoA, B, and C exhibit partial functionality in regulating SRE-dependent gene transcription. Panel (a): HEL cells were transfected with a luciferase reporter gene under control of a serum response-element-dependent promoter (pSRE-Luc), and were co-transfected with either empty vector, or the indicated RhoA, B, or C expression vectors. All Rho constructs contained valine substituted for glycine 14, rendering them constitutively active (GTP-bound); the unprenylated versions additionally contained serine substituted for cysteine 190 (RhoA and C) or 193 (RhoB). Cells were cultured in the absence (gray and open bars) or presence (black bars) of 10 μM lovastatin for 48 h. Gray and black bars represent cells transfected with single mutant Rho^{V14}; open bars represent cells transfected with double mutant $RhoA^{V14/S190}$, $RhoB^{V14/S193}$, and $RhoC^{V14/S190}$. Luciferase activities were normalized to the activity found in untreated cells transfected with pSRE-Luc plus empty vector. Panel (b): HEL cells were transfected with empty vector (lane 1), or epitope-tagged Rho constructs as described in panel (a) (lane 2 for single mutant Rho^{V14}, lane 3 for double mutant $RhoA^{V14/S190}$, $RhoB^{V14/S193}$, and $RhoC^{V14/S190}$). Cell lysates were analyzed by Western blotting with anti-epitope-specific antibodies.

leukemia generates a MKL1 fusion protein with increased transcriptional activity, suggesting that activation of SRE-dependent genes may contribute to leukemogenesis [29]. To compare the effects of prenylated versus unprenylated Rho-GTP on SRE-dependent transcription, we used constitutively-active, GTP-bound forms of RhoA, B, and C with valine substituted for glycine 14; the unprenylated proteins additionally contained serine substituted for cysteine 190, or 193 in the case of RhoB [4,18].

$RhoA^{V14}$ stimulated transcription from a SRE-luciferase reporter about 25-fold in the absence of lovastatin, and eight-fold in the presence of lovastatin; the unprenylated double mutant $RhoA^{V14/S190}$ stimulated transcription about five-fold in the absence of lovastatin (Fig. 6a). Similarly, transcriptional effects of $RhoB^{V14}$ and $RhoC^{V14}$ were reduced by lovastatin, and unprenylated $RhoB^{V14/S193}$ and $RhoC^{V14/S190}$ had less activity compared to their prenylated counterparts, but similar activities as those observed with $RhoB^{V14}$ and $RhoC^{V14}$ in lovastatin-treated cells. Expression of the prenylated and unprenylated Rho constructs was comparable, and did not explain the differences in transcriptional activation (Fig. 6b). Thus, unprenylated Rho-GTP exhibited partial transcriptional activity.

4. Discussion

Statins were originally developed as inhibitors of cholesterol synthesis, but they have pleiotropic, cholesterol-independent effects. Many of these latter effects have been attributed to inhibition of Ras and Rho signaling, and a potential role for statins in cancer prevention and therapy has been suggested [9,30]. We found that prenylation of RhoA and C was almost completely inhibited in HEL cells treated with 10 μM lovastatin, whereas N- and K-Ras farnesylation was not inhibited, suggesting that farnesylation was less affected than geranylgeranylation. In other cell types, 10–25 μM lovastatin decreased RhoA and Rac1 membrane association without affecting membrane-bound Ras, suggesting a differential effect of statins on prenylation of Rho and Ras proteins [31,32]. This may be because farnesyl transferase (EC2.5.1.58) has a K_m for farnesyl diphosphate of $\leq$5 nM, whereas geranylgeranyl diphosphate synthase (EC2.5.1.29, the enzyme which produces the substrate for geranyl-geranyl transferase, EC2.5.1.59) has a K_m of about 1 μM for the same substrate [30]. Thus, when intracellular isoprenoids are reduced by statins, geranyl-geranylation would be more severely affected than farnesylation. These data likely explain why a significant fraction of RhoB remained prenylated in lovastatin-treated HEL cells, because, unlike RhoA and C, RhoB is a substrate for farnesyl transferase, although it is not as efficiently farnesylated as Ras [33].

Lovastatin increased RhoA protein levels several-fold, and increased RhoB and C to an even greater extent, but total amounts of RhoB and C remained less than one-tenth that of RhoA. Experiments with cycloheximide showed that the statin-induced Rho accumulation required de novo protein synthesis. In addition, knock-down of RhoB mRNA with specific siRNA oligoribonucleotides prevented lovastatin-induced RhoB accumulation, suggesting a requirement for de novo mRNA and/or protein synthesis (similarly, transfection of RhoA- or RhoC-specific siRNAs prevented RhoA or RhoC accumulation, respectively; unpublished results). These results are in agreement with the findings of others who attributed the statin-induced increases in RhoA and B protein to increased mRNA transcription, increased de novo protein

synthesis, and decreased degradation of the unprenylated proteins [28], but we are unaware of previous studies quantifying statin effects on RhoA, B, and C in the same cell type.

When assessed in vitro, Rho prenylation affects RhoA activation by GEFs, RhoA association with RhoGDI, and Rho de-activation by Rho GTPase-activating proteins (RhoGAPs) [5,23,34]. Therefore, it is difficult to predict how inhibition of Rho prenylation in vivo will affect the activation state of Rho. The prevailing view is that statins inhibit Rho signaling by preventing Rho membrane association, and block agonist-induced Rho activation [12–14,30,35,36]. These studies generally examined short-term (<6 h) effects of statins on acute, GEF-mediated Rho activation. In contrast, we examined longer-term (24–48 h) effects of statins, and found that lovastatin induced a several-fold increase in total Rho-GTP, and that the increase in GTP occurred on unprenylated Rho. Longer-term statin treatment of cardiac myofibroblasts and endothelial cells increased Rho-GTP and Rac-GTP levels, respectively, but the mechanism of this increase was not examined [16,37]. While this work was in progress, Cordle et al. [15] reported that treating THP-1 monocytic cells with statins for 18 h increased Rac- and Rho-GTP levels, and that the effect on Rac could be prevented when cells were incubated with mevalonate or geranyl-geranylpyrophosphate to bypass the statin block and reconstitute Rac prenylation.

We propose that lovastatin increases Rho-GTP, at least in part, by decreasing Rho binding to RhoGDI. In vitro, binding of unprenylated Rho to RhoGDI is weak [24], and we showed that Rho prenylation is required for formation of stable Rho-RhoGDI complexes in intact cells. Using siRNA-mediated knock-down of RhoGDIα, we also demonstrated that decreased Rho-RhoGDI association is sufficient to increase Rho-GTP levels. Phosphorylation of RhoGDI can increase Rho activation, possibly by dissociating the Rho-RhoGDI complex [2]. Our finding of decreased RhoA protein in RhoGDIα-deficient cells suggests that RhoGDI binding to prenylated Rho stabilizes Rho and protects it from proteolysis, as occurs for Rac [22]. Increased Rho in lovastatin-treated cells required de novo protein synthesis leading to the accretion of unprenylated Rho proteins, which did not bind to RhoGDIs; unprenylated RhoA and B have been shown previously to be more stable than their prenylated counterparts [28], explaining their accumulation in the absence of RhoGDI binding. Increased accumulation of unprenylated Rho-GTP may be due to spontaneous GTP exchange in the absence of RhoGDI binding; alternatively, GEFs could act on unprenylated Rho, although this occurs only to a limited extent in vitro [34]. Whether RhoGAP acceleration of Rho-GTP hydrolysis is influenced by Rho prenylation is controversial [5,23].

It is generally assumed that unprenylated Rho proteins cannot interact with down-stream effectors [6]. In yeast, the unprenylated form of Ras2 has more than 100-fold lower affinity for its effector adenylate cyclase compared to the prenylated form [38], but in mammalian cells, unprenylated Ras may form inactive complexes with its effector Raf-kinase [39]. We found that unprenylated RhoA, B, and C had some transcriptional activity in HEL cells, albeit significantly less than their prenylated counterparts. Thus, unprenylated Rho appears to interact with effectors that stimulate SRE-dependent transcription, which may include Rho-kinase and protein kinase C-related kinases [18], but the interaction is less productive than with prenylated Rho.

In conclusion, we found that lovastatin markedly increased GTP-bound, unprenylated RhoA, B, and C in HEL cells, and demonstrated that the molecular mechanism of the increase involved lack of association of unprenylated Rho with RhoGDI. The functionality of unprenylated Rho-GTP may depend on the repertoire of Rho effectors present in specific cell types, and has important implications for the pleiotropic, cholesterol-independent effects of statins.

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REFERENCES

[1] Sahai E, Marshall CJ. Rho-GTPases and cancer. Nat Rev Cancer 2002;2:133–42.
[2] Dovas A, Couchman JR. RhoGDI: multiple functions in the regulation of Rho family GTPase activities. Biochem J 2005;390:1–9.
[3] Chen JC, Zhuang S, Nguyen TH, Boss GR, Pilz RB. Oncogenic Ras leads to Rho activation by activating the mitogen-activated protein kinase pathway and decreasing Rho-GTPase-activating protein activity. J Biol Chem 2003;278:2807–18.
[4] Adamson P, Marshall CJ, Hall A, Tilbrook PA. Post-translational modifications of $p21^{rho}$ proteins. J Biol Chem 1992;267:20033–8.
[5] Hori Y, Kikuchi A, Isomura M, Katayama M, Miura Y, Fujioka H, Kaibuchi K, Takai Y. Post-translational modifications of the C-terminal region of the *rho* protein are important for its interaction with membranes and the stimulatory and inhibitory GDP/GTP exchange proteins. Oncogene 1991;6:515–22.
[6] Solski PA, Helms W, Keely PJ, Su L, Der CJ. RhoA biological activity is dependent on prenylation but independent of specific isoprenoid modification. Cell Growth Differ 2002;13:363–73.
[7] Allal C, Favre G, Couderc B, Salicio S, Sixou S, Hamilton AD, Sebti SM, Lajoie-Mazenc I, Pradines A. RhoA prenylation is required for promotin of cell growth and transformation and cytoskeleton organization but not for induction of serum response element transcription. J Biol Chem 2000;275:31001–8.
[8] Wang DA, Sebti SM. Palmitoylated cysteine 192 is required for RhoB tumor-suppressive and apoptotic activities. J Biol Chem 2005;280:19243–9.
[9] Wong WW, Dimitroulakos J, Minden MD, Penn LZ. HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis. Leukemia 2002;16:508–19.

[10] Liao JK, Laufs U. Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol 2005;45:89–118.
[11] Thibault A, Samid D, Tompkins AC, Figg WD, Cooper MR, Hohl RJ, Trepel J, Liang B, Patronas N, Venzon DJ, Reed E, Myers CE. Phase I study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer. Clin Cancer Res 1996;2:483–91.
[12] Eto M, Kozai T, Cosentino F, Joch H, Luscher TF. Statin prevents tissue factor expression in human endothelial cells: role of Rho/Rho-kinase and Akt pathways. Circulation 2002;105:1756–9.
[13] del Real G, Jimenez-Baranda S, Mira E, Lacalle RA, Lucas P, Gomez-Mouton C, et al. Statins inhibit HIV-1 infection by down-regulating Rho activity. J Exp Med 2004;200:541–7.
[14] Kusama T, Mukai M, Ayaki M, Imamura F, Tatsuta M, Matsumoto Y, et al. Inhibition of lysophosphatidic acid-induced RhoA activation and tumor cell invasion by 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors. Int J Oncol 2003;23:1173–8.
[15] Cordle A, Koenigsknecht-Talboo J, Wilkinson B, Limpert A, Landreth G. Mechanisms of statin-mediated inhibition of small G-protein function. J Biol Chem 2005;280:34202–9.
[16] Porter KE, Turner NA, O'Regan DJ, Balmforth AJ, Ball SG. Simvastatin reduces human atrial myofibroblast proliferation independently of cholesterol lowering via inhibition of RhoA. Cardiovasc Res 2004;61:745–55.
[17] Ren X-D, Kiosses WB, Schwartz MA. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J 1999;18:578–85.
[18] Gudi T, Chen JC, Casteel DE, Seasholtz TM, Boss GR, Pilz RB. cGMP-dependent protein kinase inhibits serum response element-dependent transcription by inhibiting Rho activation and functions. J Biol Chem 2002;277:37382–93.
[19] Jakobisiak M, Bruno S, Skierski JS, Darzynkiewicz Z. Cell cycle-specific effects of lovastatin. Proc Natl Acad Sci USA 1991;88:3628–32.
[20] Bordier C. Phase separation of integral membrane proteins in triton X-114 solution. J Biol Chem 1981;256: 1604–7.
[21] Zeng Y, Zhuang S, Gloddek J, Tseng CC, Boss GR, Pilz RB. Regulation of cGMP-dependent protein kinase expression by Rho and Kruppel-like transcription factor-4. J Biol Chem 2006;281:16951–6.
[22] Zhang B, Zhang Y, Dagher MC, Shacter E. Rho GDP dissociation inhibitor protects cancer cells against drug-induced apoptosis. Cancer Res 2005;65:6054–62.
[23] Molnar G, Dagher MC, Geiszt M, Settleman J, Ligeti E. Role of prenylation in the interaction of Rho-family small GTPases with GTPase activating proteins. Biochemistry 2001;40:10542–9.
[24] Hancock JF, Hall A. A novel role for RhoGDI as an inhibitor of GAP proteins. EMBO J 1993;12:1915–21.
[25] Michaelson D, Silletti J, Murphy G, D'Eustachio P, Rush M, Philips MR. Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. J Cell Biol 2001;152:111–26.
[26] Gorvel JP, Chang TC, Boretto J, Azuma T, Chavrier P. Differential properties of D4/LyGDI versus RhoGDI: phosphorylation and rho GTPase selectivity. FEBS Lett 1998;422:269–73.
[27] Zalcman G, Closson V, Camonis J, Honore N, Rousseau-Merck MF, Tavitian A, et al. RhoGDI-3 is a new GDP dissociation inhibitor (GDI). Identification of a non-cytosolic GDI protein interacting with the small GTP-binding proteins RhoB and RhoG. J Biol Chem 1996;271:30366–74.
[28] Holstein SA, Wohlford-Lenane CL, Hohl RJ. Consequences of mevalonate depletion. Differential transcriptional, translational, and post-translational up-regulation of Ras, Rap1a, RhoA, and RhoB. J Biol Chem 2002;277:10678–82.
[29] Cen B, Selvaraj A, Burgess RC, Hitzler JK, Ma ZG, Morris SW, et al. Megakaryoblastic leukemia 1, a potent transcriptional coactivator for serum response factor (SRF), is required for serum induction of SRF target genes. Mol Cell Biol 2003;23:6597–608.
[30] Winter-Vann AM, Casey PJ. Opinion – post-prenylation-processing enzymes as new targets in oncogenesis. Nat Rev Cancer 2005;5:405–12.
[31] Lewis KA, Holstein SA, Hohl RJ. Lovastatin alters the isoprenoid biosynthetic pathway in acute myelogenous leukemia cells in vivo. Leuk Res 2005;29:527–33.
[32] Zhong WB, Wang CY, Chang TC, Lee WS. Lovastatin induces apoptosis of anaplastic thyroid cancer cells via inhibition of protein geranylgeranylation and de novo protein synthesis. Endocrinology 2003;144:3852–9.
[33] Lebowitz PF, Casey PJ, Prendergast GC, Thissen JA. Farnesyltransferase inhibitors alter the prenylation and growth-stimulating function of RhoB. J Biol Chem 1997;272:15591–4.
[34] Orita S, Kaibuchi K, Kuroda S, Shimizu K, Nakanishi H, Takai Y. Comparison of kinetic properties between two mammalian *ras* p21 GDP/GTP exchange proteins, *ras* guanine nucleotide-releasing factor and *smg* GDP dissociation stimulator. J Biol Chem 1993;268:25542–6.
[35] Ohkawara H, Ishibashi T, Sakamoto T, Sugimoto K, Nagata K, Yokoyama K, et al. Thrombin-induced rapid geranylgeranylation of RhoA as an essential process for RhoA activation in endothelial cells. J Biol Chem 2005;280:10182–8.
[36] Yokoyama K, Ishibashi T, Ohkawara H, Kimura J, Matsuoka I, Sakamoto T, et al. HMG-CoA reductase inhibitors suppress intracellular calcium mobilization and membrane current induced by lysophosphatidylcholine in endothelial cells. Circulation 2002;105:962–7.
[37] Vecchione C, Brandes RP. Withdrawal of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors elicits oxidative stress and induces endothelial dysfunction in mice. Circ Res 2002;91:173–9.
[38] Kuroda Y, Suzuki N, Kataoka T. The effect of posttranslational modifications on the interaction of Ras2 with adenylyl cyclase 1. Science 1993;259:683–6.
[39] Lerner E, Qian Y, Blaskovich M, Fossum R, Vogt A, Sun J, et al. Ras CAAX peptidomimetic FTI-277 selectively blocks oncogenic Ras signaling by inducing cytoplasmic accumulation of inactive Ras-Raf complexes. J Biol Chem 1995;270(45):26802–6.