Integrin-dependent Leukocyte Adhesion Involves Geranylgeranylated Protein(s)*

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Integrin-dependent leukocyte adhesion is modulated by alterations in receptor affinity or by post-receptor events. Pretreatment of Jurkat T-cells with the 3-hydroxymethylglutaryl-coenzyme A reductase inhibitor, lovastatin, markedly reduced (IC_{50} \sim 1–2 \mu M) \alpha\beta_{1}-dependent adhesion to fibronectin (FN) stimulated by phorbol 12-myristate 13-acetate (PMA) which modulates post-receptor events. In contrast, lovastatin did not inhibit Jurkat cell adhesion to FN induced by the \beta_{1} integrin-activating monoclonal antibody (mAb) 8A2, which directly modulates \beta_{1} integrin affinity. Similarly, pre-treatment of U937 cells with lovastatin inhibited PMA-stimulated, but not mAb 8A2-stimulated, \alpha\beta_{1}-dependent leukocyte adhesion to laminin. The inhibition of lovastatin on PMA-stimulated leukocyte adhesion was not mediated by mitogen-activated protein kinase or phosphatidylinositol 3-kinase pathway. The inhibitory effect of lovastatin on PMA-stimulated leukocyte adhesion was reversed by co-incubation with geranylgeraniol, but not with farnesol, with concurrent reversal of the inhibition of protein prenylation as shown by protein RhoA geranylgeranylation. The selective inhibition of protein geranylgeranylation by the specific protein geranylgeranyltransferase-I inhibitor, GGTI-298, blocked PMA-stimulated leukocyte adhesion but not mAb 8A2-induced leukocyte adhesion. The protein farnesyltransferase inhibitor, FTI-277, had no effect on leukocyte adhesion induced by either stimulus. These results demonstrate that protein geranylgeranylation, but not farnesylation, is required for integrin-dependent post-receptor events in leukocyte adhesion.

The essential role of leukocyte integrin receptors in cell-cell and cell-substrate adhesion in the inflammatory and immune systems is well established. The adhesive capacity of leukocyte integrins is highly regulated. Integrin receptors in low adhesive state do not mediate strong adhesion to other cells or ligands. However, when leukocytes are appropriately activated there is often a detectable increase in integrin adhesiveness within a few seconds to minutes. Some activation stimuli induce a measurable change in integrin receptor affinity, whereas others mediate their effects by post-receptor events such as cytoskeleton-dependent clustering of receptors that serve to increase overall avidity (1, 2). For example, the functional activity of \beta_{1} integrins on human T-cells can be regulated by treatment with certain divalent cations or activating monoclonal antibody (mAbs)† that directly increase the affinity of \beta_{1} integrins for their ligands, presumably by altering receptor conformation (3, 4). In contrast, the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), generally promotes adhesion by targeting events that occur following receptor occupancy without significantly affecting the affinity of the receptor for the ligand (3, 4). For a given leukocyte cell type, different activation stimuli may modulate integrin adhesivity by one or the other mechanism.

† A number of cytoplasmic protein regulators of integrin adhesiveness have been identified and characterized (5), the signal transduction pathways involved in the regulation of adhesiveness are still not fully elucidated. Several studies, however, have implicated the involvement of Ras family proteins. By using Chinese hamster ovary (CHO) cells transfected with \beta_{1} integrins, Hughes et al. (6) reported that overexpression of Ha-Ras suppressed increases in integrin affinity and that suppression by Ha-Ras correlated with activation of the MAP kinase pathway. These results suggested that the Ha-Ras-linked MAP kinase pathway mediated a negative feedback loop for integrin affinity modulation. Zhang et al. (7) showed that R-Ras promoted the ligand-binding activity of \beta_{1} integrins in CHO cells. The small GTP-binding protein RhoA has also been shown to be required for stimulated integrin adhesiveness in some leukocytes (8, 9). More recently, D’Souza-Schorey et al. (10) reported that the Rho subfamily protein, Rac, regulated integrin-mediated spreading and increased adhesion of T-lymphocytes.

‡ Ras-related small G-proteins, which regulate cell growth, proliferation, and differentiation, are modified by farnesyl or geranylgeranyl groups. This isoprenylation is required for their translocation to the plasma membrane and function (11, 12). These two post-translational modifications are catalyzed by the enzymes farnesyltransferase (FTase) and type I geranylgeranyltransferase (GGTase I) which recognize proteins that end

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The abbreviations used are: mAb, monoclonal antibody; CHO, Chinese hamster ovary; EEME, Earle’s modified Eagle’s medium; ERK, extracellular signal-regulated kinase; FN, fibronectin; FOH, trans-farnesol; FPP, farnesyl pyrophosphate; FTI, farnesyltransferase inhibitor; GGOH, geranylgeraniol; GGPP, geranylgeranyl pyrophosphate; GGTI, geranylgeranyltransferase inhibitor; LN, laminin; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK1, MAP kinase/ERK kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; GGTase I, type I geranylgeranyltransferase; PAGE, polyacrylamide gel electrophoresis; FTase, farnesyltransferase.
with the motif CaaX at their carboxyl termini (C is cysteine, a is aliphatic amino acid, and X is any amino acid). FTase prefers proteins that end with X as serine or methionine, whereas GGTagse I prefers X as leucine or isoleucine. Ha-Ras, having the carboxyl-terminal CVLS (the aliphatic amino acids (aa) are valine and leucine, X is serine), is selectively farnesylated, whereas Rho and Rac family GTPases have leucine at a carboxyl-terminal X position and are geranylgeranylated (13, 14). K-Ras in which X is methionine is naturally farnesylated but becomes geranylgeranylated when FTase is inhibited (15, 16).

The prenylation status of R-Ras is unclear, but its carboxylterminal sequence, CVLL, suggests that it would be geranylgeranylated. This is also supported by the fact that FTase inhibitors do not inhibit R-Ras-induced transformation (17).

In the present study we have investigated the role of protein prenylation in integrin-dependent Jurkat and U937 leucocyte adhesion by utilizing lovastatin and prenyltransferase inhibitors. Lovastatin and related drugs in this class are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, an early and rate-limiting enzyme in the sterol synthesis pathway (18). In addition to lowering cholesterol, lovastatin reduces the level of isoprenoids including geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) by depleting cellular pools of the precursors. FPP is a substrate for protein farnesytransferase and GGPP is a substrate for protein geranylgeranytransferase.

Notably, recent in vitro studies have demonstrated that a statin decreased CD11b expression and reduced CD11b-dependent adhesion of monocytes to endothelium (19), decreased ICAM-1 and LFA-1 expression in monocytes (20), and reduced leucocyte-endothelium interactions in vivo (21). We found that lovastatin inhibited PMA-stimulated adhesion, a post-receptor event, but not adhesion induced by an activating mAb that directly modulates β2 integrin affinity. Inhibition of PMA-stimulated cell adhesion by lovastatin was reversed by co-incubation with geranylgeraniol but not farnesol and correlated with inhibition of geranylgeranylation of proteins, such as RhoA. Moreover, the protein geranylgeranytransferase-I inhibitor, GGTI-298 (22), but not farnesytransferase inhibitor, FTI-277 (22), also inhibited PMA-stimulated leucocyte adhesion. These results demonstrate that a geranylgeranylated protein(s) is required for PMA-stimulated, integrin-dependent leucocyte adhesion and suggest potential new targets for small molecule inhibitors of leucocyte adhesion.

MATERIALS AND METHODS

Cell Culture—Jurkat and U937 cells (ATCC, Walkersville, MD) were maintained in Earle’s modified Eagle’s medium (EMEM, BioWhittaker, Walkersville, MD) and supplemented with 10% fetal bovine serum (HyClone Sterile System, Logan, UT), 5% glutamine (Life Technologies, Inc.), 5% sodium pyruvate (Life Technologies, Inc.), and 5 mM non-essential amino acids (Life Technologies, Inc.).

Adhesion Assay—Leucocyte adhesion to FN or LN was performed as follows. 1.25 μg/ml human FN (Collaborative Research, Inc., Bedford, MA) or 100 μg/ml mouse LN (Life Technologies, Inc.) was added to 96-well Pro-Bind™ assay plate (Falcon, Becton Dickinson, Lincoln Park, NJ) by incubating overnight at 4 °C. The plate was then blocked with 3% bovine serum albumin in phosphate-buffered saline at room temperature for 1 h. Immediately before use, plates were washed three times with phosphate-buffered saline. Jurkat cells and U937 cells were pretreated with or without lovastatin for 2.5 days in EMEM. After centrifugation for 7 min at 300 × g, cells were resuspended in 1 ml of phenol red-free medium and then labeled by incubation with 5 μl of the fluorescent dye calcein-AM (1 mg/ml in Me2SO, Molecular Probes, Eugene, OR) for 30 min at room temperature in the dark. The cells were then washed twice with phenol red-free medium. After incubation with PMA (100 ng/ml) or mAb SA2 (2 μg/ml) for 30 min in control medium at room temperature, cells (1 × 106/well) were added to triplicate wells. After incubation for 30 min at 37 °C, the total population of cells in the well was analyzed using a fluorescence plate reader (Perspective Biosystems, Framingham, MA). Unbound cells were removed by washing the plate three times with phenol red-free medium, and the plate was then reanalyzed to determine fluorescence of bound cells. After subtraction of background, the percent adherence was calculated as the emission at 530 nm of bound cells divided by the absorption of total cells. For the inhibition experiments, Jurkat cells were preincubated with the MAP kinase/ERK kinase (MEK-1) inhibitor PD-98059 (23) (Calbiochem) for 30 min in medium prior to stimulation with PMA. For the rescue experiments, Jurkat cells were co-incubated with all-trans-GGOH or trans-FOH together with lovastatin for 2.5 days prior to assay. The inhibitory effect of GGTI-298 and FTI-277 was assessed in the same way.

Immunoblotting—Whole cell lysate was prepared by rapidly pelleting 2 × 106 cells and lysing for 1 min in 100 μl of SDS sample buffer. Protein from whole cell lysate were subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose paper (Intermountain Scientific Corpora
tion, Kayville, UT). After the transfer, nitrocellulose membranes were blocked overnight at 4 °C in 5% nonfat milk in a Tris-buffered saline with 0.05% Tween 20 (TBST) and then immunoblotted with the indicated antibodies. For RhoA blots, membranes were sequentially incubated with rabbit anti-RhoA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TBST at room temperature for 1 h, washed with TBST 5 times, incubated with goat anti-rabbit IgG (H + L) conjugated with horseradish peroxidase (Bio-Rad) for 1 h, and then washed with TBST 5 times. RhoA bands were visualized on x-ray film by incubation of membrane with chemiluminescence luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at room temperature for 1 min. Extracellular signal-regulated kinase (ERK1) immunoblots were prepared by sequential incubation with anti-phosphorylated ERK1 antibody (New England Biolabs, Inc., Beverly, MA) at room temperature for 3 h and with horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h. After five washings with TBST, reactive proteins were visualized by chemiluminescence as per the manufacturer’s instructions.

For lamin B and Rap1A processing assays, Jurkat and U937 cells were treated with either vehicle, lovastatin (10 μM), FTI-277 (10 μM), or GGTI-298 (10 μM) for 2.5 days. The cells were then harvested and lysed in lysis buffer (30 mM HEPES, pH 7.5, 1% Triton X-100, 10 mM glycerol, 10 mM NaCl, 5 mM MgCl2, 25 mM NaF, 1 mM EDTA, 2 mM Na3VO4, 10 μg/ml leupeptin, 25 μg/ml trypsin inhibitor, 2 mM phenylmethylsulfonyl fluoride, 6.4 mg/ml 2-nitrophenyl phosphate). The lysates were electrophoresed on a 12.5 (Rap1A) and 7% (lamin B) SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted, respectively, with an anti-phosphorylated ERK1 antibody (New England Biolabs, Inc., Beverly, MA) at room temperature for 3 h and with horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h. After five washings with TBST, reactive proteins were visualized by chemiluminescence as per the manufacturer’s instructions.

Chemicals—Lovastatin was purchased from Calbiochem and was converted to open acid form before use. Briefly, 25 mg of lovastatin was suspended in 0.5–1 ml of ethanol, and 3 ml of 0.1 M NaOH was added. The solution was heated to 50 °C for 2 h. To this heated solution, 4.5 ml of an aqueous solution containing 81 mM Na2HPO4 and 15 mM NaH2PO4 was added. The solution was heated to 60 °C for 30 min, and the pH was adjusted to 7.3 with concentrated HCl. The final concentration was determined by UV spectroscopy (ε215 = 21, 490 M−1 cm−1). The stock solution was frozen in small aliquots. Phorbol myristate acetate (Sigma) was used at 100 ng/ml. All trans-GGOH was obtained from American Radiolabeled Chemicals (St. Louis, MO). All trans-FOH was purchased from Sigma. Monoclonal antibody P4C10 (anti-β2) and P1D6 (anti-αv) were used at 1:400 dilution (Life Technologies, Inc.). Monoclonal antibody HIP1/2 (anti-αv) was a gift from Dr. Roy Lobb (Biogen, Cambridge, MA). Monoclonal antibody GoH3 (anti-α5) was obtained from Kamiya Biomedical Co. (Seattle, WA). Monoclonal antibody 5D1 (anti-β2) was a gift of B. Schwartz (University of Washington, Seattle, WA). GGTI-298 and FTI-277 were prepared as described previously (22).

RESULTS

Lovastatin Inhibits Jurkat Cell Adhesion to Fibronectin—Both PMA and the β1 integrin-activating mAb 8A2 markedly stimulated Jurkat cell adherence to FN (Fig. 1A), and this stimulated adhesion was reversed by mAb to the β2 subunit but not the αv subunit (data not shown). PMA and 8A2 also stimulated U937 cell adherence to LN (Fig. 1B), and this stimulated adhesion was blocked by mAb to the α5 subunit (data not shown). To determine the effect of lovastatin on leucocyte adhesion, we exposed the cells to varying concentrations of lovastatin for 2.5 days prior to the adherence assay. As shown
in Fig. 1A, lovastatin at concentrations greater than 5 μM nearly completely inhibited PMA-stimulated Jurkat cell adherence to FN with an IC50 of 1–2 μM. Addition of lovastatin at the time of the adhesion assay without pretreatment had no inhibitory effect (data not shown). Importantly, lovastatin had no significant effect on mAb 8A2-stimulated Jurkat cell adhesion to FN.

Lovastatin also inhibited PMA-, but not mAb 8A2-, stimulated adherence of U937 cells to LN (Fig. 1B). These results suggested that PMA-induced leukocyte adhesion is regulated by one or more products in the cholesterol biosynthesis pathway.

The Inhibitory Effect of Lovastatin on Leukocyte Adhesion Is Reversed by Co-incubation with Geranylgeraniol but Not Farnesol—Lovastatin is an inhibitor of 3-hydroxymethylglutaryl-coenzyme A reductase which catalyzes the production of mevalonic acid. Mevalonic acid can be converted to both of the two substrates for protein prenylation, GGPP and FPP. The inhibitory effect of lovastatin on stimulated leukocyte adhesion could be explained by the lowering of the cellular pool of either GGPP or FPP. In order to determine which isoprenyl group might be involved, we depleted the cellular pool of both prenylpyrophosphates by lovastatin treatment and then determined whether GGPP or FPP rescued the stimulated adhesion. Since GGOH and FOH can rescue geranylgeranylation of Rap1A and farnesylation of Ras, respectively (28), we used GGOH and FOH, which are more cell-permeable. Because the isoprenyl groups that modify small G-proteins are all-trans-isomer, all-trans-GGOH and all-trans-FOH were used. When cells were treated with lovastatin together with GGOH or FOH, GGOH (Fig. 2A and B) but not FOH (Fig. 2C) reversed the inhibitory effect of lovastatin on leukocyte adhesion. 2.5 μM GGOH was sufficient to rescue Jurkat cells from the inhibitory effect of 5 μM lovastatin (Fig. 2B). These results demonstrate that geranylgeraniol but not farnesol is required for PMA-stimulated leukocyte adhesion.

Lovastatin Inhibits Protein Prenylation—Several Ras family members, Hu-Ras, R-Ras, RhoA, and Rac, have been implicated in cell adhesion (9, 10, 14, 25–27). RhoA and Rac are gera-
nlygeranylated proteins (9, 10, 28). Because RhoA (8, 9), but not Rac (10), was reported to be involved in PMA-stimulated leukocyte adhesion, we next examined the effect of lovastatin on RhoA isoprenylation and the correlation between RhoA isoprenylation and leukocyte adhesion. After co-incubation for 2.5 days, lovastatin significantly inhibited the geranylgeranylation of RhoA at concentrations as low as 0.5 μM. The increasing ratio between the intensity of apparently higher molecular weight RhoA protein and the apparently lower molecular weight RhoA protein with increasing concentration of lovastatin reflects decreasing isoprenylation of protein as the nonprenylated Rho protein migrates more slowly than prenylated Rho protein (29). WithoutLovastatin, the majority of RhoA in cells was isoprenylated. At 14 μM lovastatin, most RhoA was in non-isoprenylated form (Fig. 3), and at this concentration stimulated leukocyte adhesion to FN was completely inhibited. Addition of GGGOH (5 μM) with lovastatin resulted in the more rapid migration of Rho as seen in untreated cells (data not shown). These results suggest that geranylgeranylation of a protein(s) such as RhoA is required for PMA-stimulated Jurkat cell adhesion.

GGTI-298, but Not FTI-277, Inhibits Leukocyte Adhesion in a Dose- and Time-dependent Manner—Since the inhibitory effect of lovastatin was reversed by co-incubation with GGGOH and since lovastatin inhibited protein geranylgeranylation, we next tested the effect of inhibitors that directly block protein prenylation. We first evaluated the ability of FTI-277 and GGTI-298 to inhibit protein farnesylation and geranylgeranylation, respectively, in Jurkat and U937 cells. To this end, we treated these cells with either vehicle, lovastatin, FTI-277, or GGTI-298 and processed the samples for immunoblotting with antibodies against lamin B and Rap1A as described under “Materials and Methods.” Fig. 4 shows that treatment with FTI-277 resulted in inhibition of lamin B processing as indicated by the band shift. GGTI-298, on the other hand, had no effect on lamin B processing. Fig. 4 also shows that GGTI-298 inhibited the processing of the geranylgeranylated small G-protein Rap1A. In contrast, FTI-277 had no effect on Rap1A processing. Thus, in both Jurkat and U937 cells GGTI-298 inhibited the processing of Rap1A but not lamin B. Similarly, FTI-277 inhibits lamin B but not Rap1A processing. The results of Fig. 4 clearly demonstrate that GGTI-298 and FTI-277 were selective for their intended targets, GGTase I and FTase, respectively. As expected, lovastatin inhibited the processing of both lamin B and Rap1A but was much more potent at inhibiting Rap1A than lamin B (Fig. 4).

We next examined the effects of FTI-277 and GGTI-298 on leukocyte adhesion. Fig. 5, A and B, shows that GGTI-298 inhibited PMA-induced U937 cell adhesion to LN in a dose- and time-dependent manner. The IC50 was ~2–3 μM which is in agreement with the effect of this inhibitor on protein geranylgeranylation in vivo (22). At this concentration protein farnesylation is not inhibited by GGTI-298. One day pretreatment with 10 μM GGTI-298 produced ~50% inhibition of cell adhesion, consistent with the half-life of RhoA (28). FTI-277 did not inhibit PMA-induced adhesion of either U937 or Jurkat cells (Fig. 5, C and D). Fig. 4 shows that FTI-277 does not inhibit protein geranylgeranylation, but GGTI-298 does at the condition we are using. These indicate that protein farnesylation is not required for stimulated leukocyte adhesion. Together with the rescue experiments, these results demonstrate that a geranylgeranylated protein(s) is involved in the signaling pathway, which regulates integrin adhesiveness.

Lovastatin Does Not Inhibit PMA-stimulated MAPK Phosphorylation—To establish that lovastatin did not inhibit a proximal component of PMA-stimulated activation, i.e. its effect on PMA-induced adhesion was not due to blockade of all PKC-mediated signaling, we determined its effect on PMA-stimulated MAPKK activation. ERK1 and ERK2 activation is required for many signal transduction events (30). Phorbol 12-myristate 13-acetate activates PKC, and, ultimately, results in threonine and tyrosine phosphorylation of ERK1 and ERK2. Phosphorylation of ERK1 in control and lovastatin-treated cells was monitored by immunoblot analysis with an antibody specific for phosphorylated ERK1 antibody that recognizes only the phosphorylated form of ERK1. The immunoblot analysis in Fig. 6 shows that PMA still activated this MAPK cascade even at lovastatin concentrations up to 40 μM. This result suggests that treatment with lovastatin did not disable all components of PMA-stimulated signaling but specifically blocked one or more elements in the pathway leading to increased β1 integrin adhesiveness. These results also suggest that the MAPK cascade does not play a role in PMA-induced leukocyte adhesion.

The MAPK Cascade and PI-3 Kinase Pathway Are Not Involved in PMA-stimulated Cell Adherence to Fibronectin—To confirm further that the MAPK cascade does not play a role in PMA-stimulated cell adhesion, we pretreated cells with PD-98059, a specific inhibitor of MEK-1, for 30 min at room temperature prior to the adhesion assay. Treatment with PD-98059 (10 μM) did not inhibit PMA-stimulated Jurkat cell adhesion (Fig. 7A), although immunoblot analysis demonstrated that ERK1 phosphorylation was completely blocked (Fig. 7B). Pretreatment with the PI-3 kinase inhibitor LY 294002 at 14 μM also did not inhibit PMA-stimulated leukocyte adhesion (data not shown).

DISCUSSION

Integrin-dependent leukocyte adhesion is modulated by alterations in receptor affinity or by post-receptor events (1–4). Pretreatment of Jurkat T-cells with lovastatin markedly reduced α6β1-dependent adhesion to FN stimulated by PMA, which modulates post-receptor events. In contrast, lovastatin did not inhibit Jurkat cell adhesion to FN induced by the β1 integrin-activating mAb 8A2, which directly modulates β1 integrin affinity. Similarly, pretreatment of U937 cells with lovastatin inhibited PMA+, but not mAb 8A2+, stimulated α6β1-de-
Fig. 5. GGTI-298, but not FTI-277, inhibits PMA-stimulated leukocyte adhesion in a dose- and time-dependent manner. The assays in A–C were performed with LN-coated plates. A, U937 cells were treated with various concentrations of GGTI-298 for 2.5 days. B, U937 cells were treated with 10 μM GGTI-298 for various amounts of time. C, U937 cells were treated with 10 μM FTI-277 for 2.5 days. D, Jurkat cells were treated with 1 μM FTI-277 for 2.5 days and then assessed for adhesion to FN-coated plates.

Independent leukocyte adhesion to LN. Flow cytometry analysis showed that treatment with lovastatin did not alter the integrin expression on U937 or Jurkat cell surface (not shown), indicating that the inhibitory effect of lovastatin on cell adhesion was not due to a reduction of receptor number. Thus, lovastatin inhibited PMA-stimulated adhesion of two different β1 integrin receptors, in two different leukocyte cell lines, by targeting signaling pathways regulating post-receptor events.
but not by inhibiting the integrin expression on the cell surface.

Recently, several proteins involved in signal transduction have been shown to be lipid-modified by covalent attachment of farnesyl or geranylgeranyl groups, which are derived from mevalonic acid (31). Lovastatin inhibits the biosynthesis of both of these isoprenoids. Thus, one possible mechanism by whichLovastatin inhibits leukocyte adhesion is interference with a signaling pathway(s) that requires an isoprenylated protein(s). The fact that all-trans-GGOH, but not FOH, prevented Lovastatin-induced inhibition of leukocyte adhesion suggested that a protein(s) modified by a geranylgeranyl-group, not a farnesyl-group, was required for PMA-stimulated leukocyte adhesion. However, since Lovastatin targets an early step in the sterol synthesis pathway, Lovastatin might also inhibit the biosynthesis of other lipid moieties, which are required for leukocyte adhesion (Fig. 8). Although the rescue by GGOH and the inhibition of RhoA geranylgeranylation strongly suggested that a geranylgeranylated protein(s) regulated leukocyte adhesion, it remained possible that GGOH itself or a derivative, like vitamin Q (also called ubiquinone) which can be converted from geranyleranyl pyrophosphate, regulates cell adhesion (Fig. 8). Also, integrin-modulating factor-1, possibly an isoprenyl lipid, has been reported to regulate integrin function (32). GG motif demonstrates thatLovastatin and GGTI-298 inhibit PMA-stimulated adhesion without changing cellular lipid content by targeting protein geranylgeranyltransferase-I. Thus, the inhibitory effect of GGTI-298 demonstrates a geranylgeranylated protein(s) indeed regulates post-receptor events in PMA-stimulated leukocyte adhesion (Fig. 8). Whether this geranylgeranylated protein(s) regulates kinases (e.g. RhoA kinase), the synthesis of integrin-modulating lipids, or other signaling pathways remains to be determined. The fact that the specific PTase inhibitor, FTI-277, did not inhibit leukocyte adhesion confirmed the rescue experiments showing that farnesylated proteins are not involved (Fig. 8).

Ras family proteins require prenylation for function, and several have been implicated in the regulation of integrin function. Ha-Ras was shown to suppress integrin affinity in CHO cells through activation of the MAPK pathway (6). Our studies demonstrate thatLovastatin and GGTI-298 inhibit PMA-stimulated adhesion, which involves post-receptor events rather than affinity modulation (1–4). Moreover, Ha-Ras requires farnesylation for function (33), whereas PMA-stimulated adhesion requires a geranylgeranylated protein(s). Furthermore, inhibition of the MAPK pathway did not affect PMA-stimulated adhesion. Ha-Ras is therefore not involved in our model system.

In contrast to Ha-Ras, R-Ras, was shown to promote integrin-dependent adhesion in CHO cells (7). However, the effect of R-Ras again involved increases in receptor affinity rather than post-receptor events. Also, R-Ras may signal through PI-3 kinase (34), but the PI-3 kinase inhibitor LY 294002 had no effect on PMA-stimulated leukocyte adhesion. Thus, although it is unlikely geranylgeranylated, R-Ras is not likely involved in PMA-stimulated leukocyte adhesion.

Rho subfamily proteins are geranylgeranylated, and two members, Rac and RhoA, have been reported to be involved in β1 integrin-dependent leukocyte adhesion (8–10). An activated mutant of Rac triggered adhesion and spreading of a T-lymphocyte line to FN, but, importantly, a dominant-negative Rac did not block PMA-stimulated spreading on FN (10). Inhibition of RhoA by C3 transferase exoenzyme inhibited PMA-stimulated β2 integrin-dependent human neutrophil adhesion to fibrinogen and αβ1-dependent murine lymphocyte cell line adhesion to VCAM-1 (8). However, C3 transferase exoenzyme did not inhibit PMA-stimulated β2 integrin-dependent lymphocyte adhesion to ICAM-1 (35). Moreover, C3 transferase exoenzyme treatment increased PMA-stimulated integrin-dependent

![Image](image-url)
monocyte and U937 cell spreading on FN (36). Thus, the role of RhoA in PMA-stimulated leukocyte adherence is uncertain. Although our studies show that reduced isoprenylation of RhoA correlated with the inhibition of PMA-stimulated adhesion by lovastatin, we cannot conclude that RhoA is the critical geranylgeranylated protein regulating post-receptor events.

It is interesting that lovastatin inhibited PMA-induced adhesion without blocking the MAPK cascade signaling pathway. PMA activates PKC which is generally considered to lie upstream of Ras (33, 37, 38), and a previous report showed that expression of a dominant-negative Ras mutant in Jurkat T-cells was able to prevent PMA-stimulated up-regulation of CD69 (33). Since Ras lies upstream of the MAPK cascade (34, 39, 40), activation of Ras will stimulate and blockade of Ras will inhibit the MAPK cascade. Ras has to be isoprenylated in order to function (28). Our study shows that at concentrations of lovastatin at which protein isoprenylation and cell adhesion were inhibited, activation of the MAPK cascade was not inhibited. A likely explanation is that PKC in fact functions downstream of Ras by activating Raf-1 kinase as previously reported (23, 41, 42). Thus, although Ras function was likely blocked by lovastatin, PKC still activated the MAPK cascade via Raf-1. Since lovastatin inhibited PMA-stimulated cell adhesion, but not MAPK activation, we surmise that in Jurkat cells the PMA-stimulated signaling pathway regulating $\beta_1$ integrin-dependent adhesion does not involve the MAPK cascade. This conclusion is supported by studies showing that the MEK-1 inhibitor PD-98059 (10 $\mu$m) potently inhibited PMA-stimulated MAPK activity but did not inhibit PMA-stimulated leukocyte adhesion.

In conclusion, lovastatin blocked PMA-stimulated leukocyte adhesion, and GGGOH but not FOH reversed this inhibitory effect. The GGTase-specific inhibitor, GGTI-298, was not an FTase-specific inhibitor, FTI-277, also blocked PMA-stimulated adhesion. The GGTase-specific inhibitor, GGTI-298, but not MAPKK activity but did not inhibit PMA-stimulated leukocyte adhesion. This is consistent with previous studies showing that FTase inhibitors block PKC activation of Raf-1 kinase and consequently inhibit PMA-stimulated cell adhesion (22, 23).

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In conclusion, lovastatin blocked PMA-stimulated leukocyte adhesion and GGOH but not FOH reversed this inhibitory effect. The GGTase-specific inhibitor, GGTI-298, but not FTase-specific inhibitor, FTI-277, also blocked PMA-stimulated leukocyte adhesion. Together, these results demonstrate that a geranylgeranylated protein(s) regulates post-receptor events in integrin-dependent PMA-stimulated leukocyte adhesion as shown in Fig. 8.