ARTHRITIS & RHEUMATISM
Vol. 54, No. 11, November 2006, pp 3441–3451
DOI 10.1002/art.22169

RhoA-Mediated, Tumor Necrosis Factor α–Induced Activation of NF-κB in Rheumatoid Synoviocytes

Inhibitory Effect of Simvastatin

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Objective. **Increasing evidence indicates that RhoA may play a central role in the inflammatory response. This study was conducted to examine the role of RhoA in mediating the activation of NF-κB in tumor necrosis factor α (TNFα)–stimulated rheumatoid synoviocytes, and to evaluate the modulatory effects of statins on the TNFα-induced activation of RhoA and NF-κB and the secretion of proinflammatory cytokines by rheumatoid synoviocytes.**

Methods. **Rheumatoid synoviocytes obtained from patients with active rheumatoid arthritis were stimulated with TNFα and incubated with simvastatin (SMV) (1 μM). RhoA activity was assessed by a pull-down assay. NF-κB DNA binding activity and nuclear translocation of NF-κB were measured by a sensitive multiwell colorimetric assay and confocal fluorescence microscopy, respectively.**

Results. **TNFα stimulation elicited a robust increase in RhoA activity in a dose-dependent manner, and SMV mitigated this increase. TNFα also hastened NF-κB nuclear translocation of subunit p65 and increased DNA binding activity, luciferase reporter gene expression, degradation of IκB, and secretion of interleukin-1β (IL-1β) and IL-6. SMV prevented the increase in NF-κB activation and rise in IL-1β and IL-6 levels induced by TNFα, whereas mevalonate and geranylgeranyl pyrophosphate reversed the inhibitory effects of SMV on activation of NF-κB and RhoA. Furthermore, cotransfection with a dominant-negative mutant of RhoA demonstrated that the TNFα-induced signaling pathway involved sequential activation of RhoA, leading to NF-κB activation and, ultimately, to secretion of cytokines.**

Conclusion. **This study identifies RhoA as the key regulator of TNFα-induced NF-κB activation, which ultimately results in the secretion of proinflammatory cytokines in rheumatoid synoviocytes. The findings provide a new rationale for the antiinflammatory effects of statins in inflammatory arthritis.**

Rheumatoid arthritis (RA) is a common chronic inflammatory disease characterized by infiltrations of macrophages and T cells into the joints, as well as synovial hyperplasia. Inflammatory cytokines have been recognized as a significant factor in the pathogenesis of RA (1). The success of anticytokine therapies in RA, particularly anti–tumor necrosis factor α (anti-TNFα) and anti–interleukin-1 (anti–IL-1), has revealed the critical pathogenetic importance of cytokines (2). However, anticytokine therapies have generally involved biologic agents that are selective for a single factor. Thus, the focus has begun to shift to the development of biologic agents that target specific signal transduction pathways, which can regulate the expression of an array of cytokines.

NF-κB seems to have a central role in mediating a variety of immune and inflammatory responses. Indeed, NF-κB has been shown to be involved in regulating the expression of genes that encode inflammatory cytokines, immune growth factors, immunoreceptors, cell adhesion molecules, and acute-phase proteins (3). In resting cells, NF-κB is sequestered in the cytoplasm and

Supported in part by grants for cooperative scientific research from First Affiliated Hospital and Life Science College of Sun Yat-sen University, China.

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Submitted for publication December 26, 2005; accepted in revised form July 17, 2006.

is therefore inhibited by members of the IκB family, including IκBα and IκBβ. Once activated, IκB proteins are phosphorylated by a complex of IκB kinases and then ubiquitinated and rapidly degraded by the proteasome, allowing NF-κB to be released from IκB and to translocate to the nucleus and initiate transcription by binding to numerous specific gene promoter elements (4,5).

There is increasing evidence to suggest that NF-κB activation participates in the pathogenesis of RA. For instance, it has been demonstrated that NF-κB activation is significantly higher in RA synovium than in osteoarthritis synovium (6,7). Furthermore, immunohistochemical analysis has demonstrated nuclear translocation of the p50 and p65 NF-κB proteins in the synovial intimal lining (7). NF-κB in cultured fibroblast-like synoviocytes is rapidly activated after stimulation by TNFα and induces production of many cytokines, such as IL-6 and IL-1β (8,9). Several animal models of inflammatory arthritis have also shown that inhibition of NF-κB in vivo can suppress joint inflammation (10–12). These findings suggest that NF-κB may be an attractive therapeutic target for RA (13).

The Rho family of small GTPases, consisting of Rho, Rac, and Cdc42, are 20- to 40-kd monomeric G proteins that can cycle between 2 interconvertible forms: the GDP-bound (inactive) state and GTP-bound (active) state (14,15). Activated Rho binds to specific downstream effectors, resulting in several cellular biologic functions, including formation of actin stress fibers, focal adhesion, cell motility, aggregation of cells, proliferation, and transcriptional regulation (15,16). There is increasing evidence to support the notion that small Rho GTPases and their exchange factors are important components of the signaling pathways used by antigen, costimulatory, cytokine, and chemokine receptors to regulate the immune response (17–20).

Rho GTPases, for example, have been implicated in the regulation of NF-κB activation and proliferation in T cells (17,20). It has been reported that signaling mediated by the Rho small GTP-binding protein promotes proliferation of rheumatoid synovial fibroblasts (21), and that the Rho pathway also mediates activation of NF-κB and expression of cytokines in TNFα-induced peripheral blood mononuclear cells (PBMCs) from patients with Crohn's disease, a chronic inflammatory disease (22). These studies indicate that the Rho pathway may play an important role in a few chronic inflammatory diseases; however, it remains unknown whether Rho GTPases mediate the inflammatory responses in RA.

The 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, or statins, are potent inhibitors of cholesterol biosynthesis that are used extensively in the treatment of patients with hypercholesterolemia (23,24). Recent studies indicate that statins may exert antiinflammatory effects and immunomodulatory activities (25). For instance, it has been reported that statins can abrogate the Th1 immune response, promote the release of Th2 cytokines, prevent the production of chemokines, and inhibit the proliferation of T cells and endothelial cells (26–29). Moreover, simvastatin (SMV) can markedly inhibit murine collagen-induced arthritis, a surrogate model for human RA, via specific suppression of the pathogenic Th1 and proinflammatory responses (30). However, the effects of statins on intracellular signaling in RA remain unknown.

It is usually assumed that the beneficial effects of statins result from the competitive inhibition of cholesterol synthesis. However, statins may exert additional beneficial effects beyond their cholesterol-lowering properties by preventing the synthesis of various isoprenoid intermediates, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which serve as lipid attachments for a variety of intracellular signaling molecules, including small GTP-binding proteins (31–33). We have also recently shown that some of the beneficial effects of statins may be mediated via modulation of the activity of the Rho GTPase (34,35).

In the present study, we examined the hypothesis that TNFα may contribute to the activation of NF-κB via a RhoA-mediated pathway in rheumatoid synoviocytes, and also postulated that statins, such as SMV, may be beneficial in RA by modulating the TNFα-induced, RhoA-mediated NF-κB signaling pathway and by preventing the enhanced secretion of cytokines induced by TNFα in rheumatoid synoviocytes.

PATIENTS AND METHODS

Reagents and antibodies. TNFα was obtained from R&D Systems (Minneapolis, MN). RPMI 1640, fetal calf serum (FCS), antibiotics, trypsin–EDTA, phosphate buffered saline (PBS), and other products for cell culture were purchased from Invitrogen (Carlsbad, CA). The collagenase, mevalonate (MEV), GGPP, and β-actin antibodies were purchased from Sigma (St. Louis, MO). The RhoA activation assay kit was obtained from Upstate Biotechnology (Lake Placid, NY). The RhoA, IκBα, and NF-κB p65 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Plasmids containing wild-type RhoA (Q63LRhoA) and dominant-negative RhoA (T19NRhoA) constructs were obtained from Upstate Biotechnology. SMV was chemically activated as described previously (34).

Cell culture and transfection. Synovial tissue specimens were obtained from patients with active RA (5 women, ages 48–69 years) whose disease was defined according to the revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (36) and who were undergoing synovectomy or joint replacement. Normal synovial tissue was obtained from healthy subjects by arthroscopic biopsy. The synovial tissue was cut into small pieces and digested with collagenase in RPMI 1640 for 2 hours at 37°C, to isolate synoviocytes. The synoviocytes were then grown in RPMI 1640 medium containing 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C under 5% CO_2. When confluence was reached, the cells were trypsinized and passaged, and used after 2–5 passages. We determined that the cultured synoviocytes were synovial fibroblast-like cells.

For transfections of RhoA mutants, cells were grown to 50–60% confluence and then transfected with 1 μg of fusion plasmid DNA using Lipofectamine reagent according to the manufacturer's instructions (Invitrogen). Subsequently, the transfected colonies were grown in growth medium containing 800 μg/ml G418 (Invitrogen) until the cells achieved 70–80% confluence.

Measurement of RhoA activity. RhoA activity was measured using a pull-down assay with the Rho-binding domain of the Rho effector protein rhotekin, in accordance with the manufacturer's instructions (Rho activity assay kit; Upstate Biotechnology). Briefly, 10^7 cells were grown in 100-mm dishes, washed twice in ice-cold PBS, and lysed in ice-cold MLB buffer (25 m*M* HEPES [pH 7.5], 150 m*M* NaCl, 1% Nonidet P40, 10 m*M* $MgCl_2$, 1.0 m*M* EDTA, and 2% glycerol). The samples were centrifuged and incubated for 45 minutes at 4°C with 10 μl of rhotekin agarose to precipitate GTP-bound Rho. Precipitated complexes were washed 3 times in MLB buffer and resuspended in 30 μl of 2× Laemmli buffer. Total samples and precipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using monoclonal antibody against RhoA at a dilution of 1:500.

Western blotting. For each Western blot experiment, 5×10^5 cells were seeded. When subconfluence (~70%) was reached, the cells were made quiescent for 48 hours and then rinsed twice with ice-cold PBS, after which 0.5 ml of ice-cold lysis buffer (50 m*M* Tris HCl [pH 7.5], 150 m*M* NaCl, 100 μg/ml phenylmethylsulfonyl fluoride, 0.1% SDS, 1% Nonidet P40, 0.5% sodium deoxycholate, 10 μg/ml aprotinin, 2 μg/ml leupeptin, and 10 m*M* EDTA) was added. Subsequently, the cells were incubated on ice for 20 minutes and then scraped and centrifuged.

Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of protein were solubilized in Laemmli buffer (62.5 m*M* Tris HCl [pH 6.8], 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.00625% bromphenol blue), boiled for 5 minutes, and then separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with primary antibodies (diluted 1:500 for RhoA, 1:500 for IκBα, and 1:5,000 for β-actin) in Tris buffered saline–Tween containing 5% nonfat milk at 4°C overnight. The membranes were incubated with the appropriate secondary antibodies for 1 hour at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Uppsala, Sweden). The blotting results shown herein represent 1 of at least 3 similar, independent experiments.

Confocal laser scanning fluorescence microscopy. For fluorescence microscopy, synoviocytes were grown on glass coverslips. The cells were fixed with acetone and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. The cells were incubated with anti–NF-κB p65 antibody (diluted 1:100) for 1 hour at room temperature, and then incubated with fluorescein isothiocyanate–conjugated secondary antibody (Santa Cruz Biotechnology). After washing in PBS, cells were incubated for 3 minutes with 0.25 mg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). The cover slips were mounted on glass slides with antifade mounting media and examined using confocal fluorescence microscopy (LSM510; Zeiss, Jena, Germany).

Measurement of NF-κB DNA binding activity. The DNA binding activity of NF-κB was measured by a sensitive multiwell colorimetric assay (37) using a TransAM NF-κB Kit (Active Motif, Carlsbad, CA). Briefly, cultured synoviocytes on culture plates were scraped and centrifuged for 10 minutes at 1,500 revolutions per minute. The pellet was resuspended in 100 μl of lysis buffer, and the lysate was centrifuged for 20 minutes at 15,000 rpm. Supernatant constituted the total protein extract. Cell extracts (5 μg) from each sample were incubated in 96-well plates coated with the NF-κB consensus, double-stranded oligonucleotide sequence (5′-AGTTGAGGGGGACTTTCCCAGGC-3′) for 1 hour and then with supplied primary NF-κB antibody (diluted 1:500) for 1 hour, and subsequently with secondary peroxidase-conjugated antibody (diluted 1:1,000) for 1 hour at room temperature. After a colorimetric reaction, the optical density was read at 450 nm. For competition assays, the cell extracts were incubated with the 22-bp double-stranded DNA, either wild-type or mutated (5′-AGTTGAGCTCACTTTCCCAGGC-3′; underline denotes the substitution).

NF-κB reporter assay. Rheumatoid synoviocytes were transiently transfected with 1 μg of pNF-κB-Luc plasmid by using Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. Twenty-four hours later, transfected cells were starved overnight in serum-free medium and then seeded in 96-well plates. Cells were then treated with different agents and harvested in reporter lysis buffer (Promega, Madison, WI). The transfection efficiency of the cells was normalized to the expression levels of β-galactosidase, and luciferase enzyme activity was then quantified using a reporter assay kit (Clontech, Palo Alto, CA).

Detection of secretion of IL-1β and IL-6. Synoviocytes, either transfected with or free of dominant-negative RhoA plasmid, were stimulated with TNFα at a concentration of 100 pg/ml for 12 hours in the presence or absence of SMV (1 μ*M*). For studying the role of NF-κB in cytokine secretion, cells were pretreated with pyrrolidine dithiocarbamate (PDTC) (300 μ*M*), an inhibitor of NF-κB activity, for 1 hour. The conditioned media were collected, and the secretion of IL-1β and IL-6 was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (R&D Systems) according to the manufacturer's instructions.

Statistical analysis. Results are expressed as the mean ± SEM. Analysis of variance with a Student-Newman-Keuls test was used to evaluate differences between ≥2

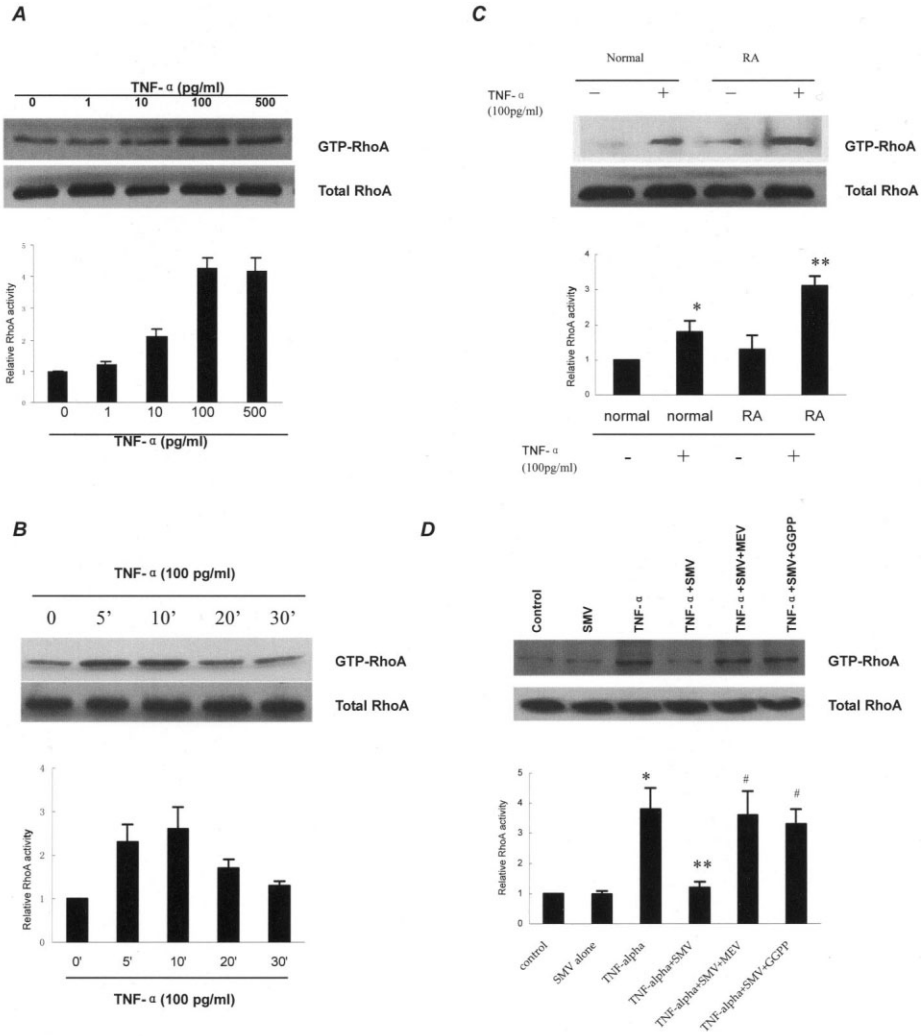

Figure 1. Effects of tumor necrosis factor α (TNFα) and simvastatin (SMV) on RhoA activity in rheumatoid synoviocytes. GTP-bound RhoA was precipitated in a pull-down assay using the fusion protein GST-RBD. **A,** Rheumatoid synoviocytes were exposed to various concentrations of TNFα for 10 minutes, and RhoA activity was assessed on polyacrylamide gels (upper panel) or measured semiquantitatively (lower panel) and shown as the mean and SEM amount of GTP-bound RhoA normalized to the amount of total RhoA in samples from 3 rheumatoid arthritis (RA) patients in 1 of 3 separate experiments. **B,** Rheumatoid synoviocytes were stimulated with 100 pg/ml TNFα for varying times, and RhoA activity was assessed on polyacrylamide gels (upper panel) or measured semiquantitatively (lower panel) and shown as the mean and SEM in 3 RA samples from 1 of 3 separate experiments. **C,** Stimulation with 100 pg/ml TNFα induced RhoA activation in both normal and RA synoviocytes (upper panel). This is also shown semiquantitatively (lower panel) as the mean and SEM in 3 normal control samples or 3 RA samples from 1 of 3 separate experiments. $* = P < 0.05$ versus nonstimulated normal synoviocytes; $** = P < 0.05$ versus TNFα-stimulated normal synoviocytes. **D,** Effect of SMV on activation of RhoA by rheumatoid synoviocytes. The cells pretreated with SMV (1 μM) for 18 hours were stimulated with TNFα (100 pg/ml) for 10 minutes. Mevalonate (MEV) (200 μM) and geranylgeranyl pyrophosphate (GGPP) (10 μM) were added for 24 hours or 8 hours, respectively, before harvest. A representative gel (upper panel) shows the results in 3 RA samples from 1 of 3 separate experiments, while bars (lower panel) show the mean and SEM RhoA activity in the 3 RA samples. $* = P < 0.05$ versus control; $** = P < 0.05$ versus TNFα; **#** $= P < 0.05$ versus TNFα + SMV.

different experimental groups. A P value less than or equal to 0.05 was considered significant.

RESULTS

Effect of TNFα and SMV on RhoA activity. To decipher the effect of TNFα on activation of RhoA (a member of the Rho family of small GTPases), rheumatoid synoviocytes were exposed to different doses of TNFα (1, 10, 100, and 500 pg/ml) for 10 minutes. As shown in Figure 1A, increases in RhoA activity occurred in a dose-dependent manner with TNFα concentrations ranging from 0 to 500 pg/ml, and without significant changes in the

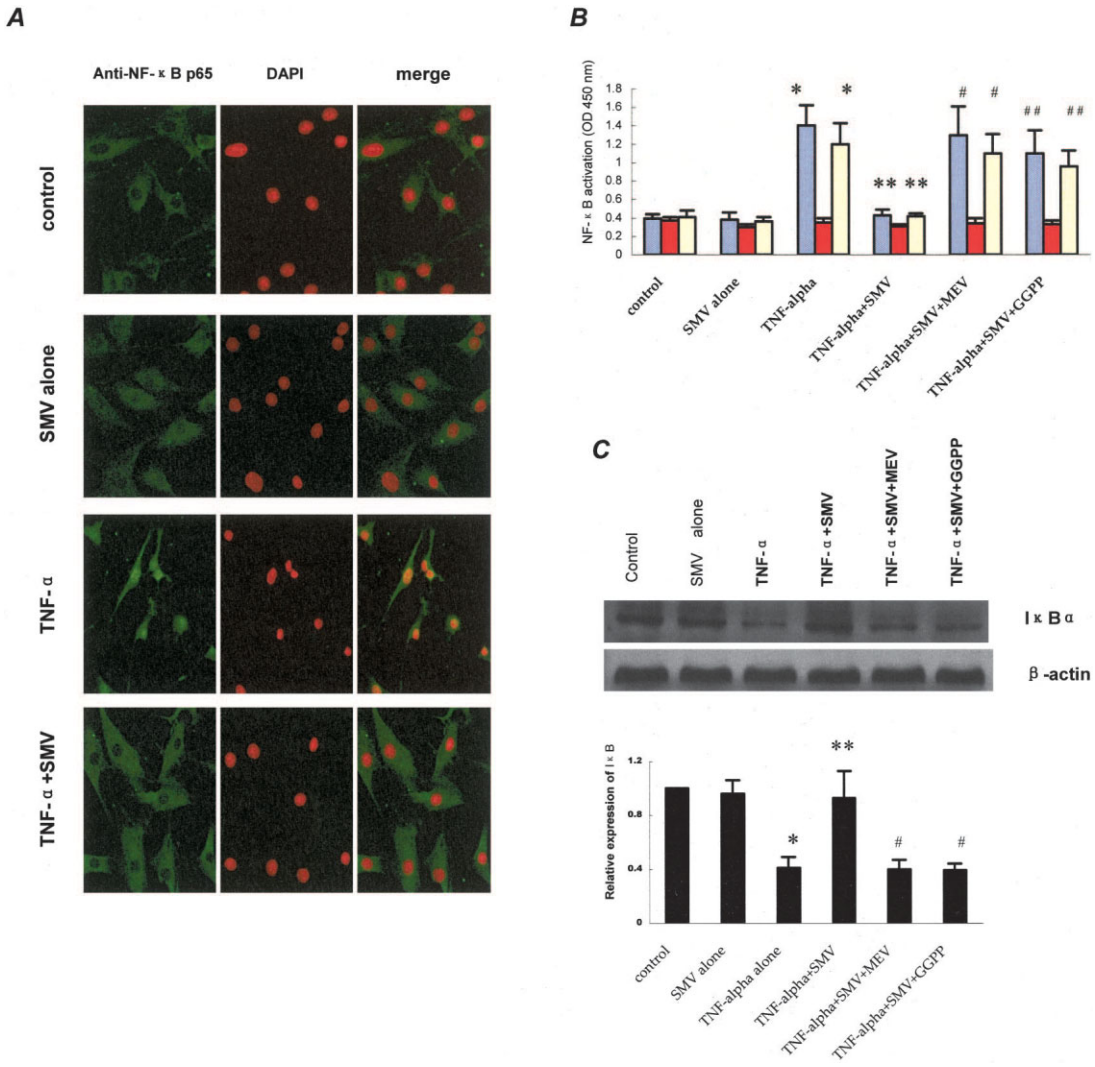

Figure 2. Effects of TNFα and SMV on NF-κB activation. **A,** The nuclear translocation of NF-κB subunit p65 was assessed by confocal fluorescence microscopy using anti-p65 antibody. Representative laser confocal microcopy images show nuclear translocation and colocalization of p65 (green stain) with nuclei stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (red stain) in cells exposed to TNFα (100 pg/ml) and/or SMV (1 μM). **B,** Cells were left untreated or were pretreated for 18 hours with SMV (1 μM) and/or stimulated with TNFα (100 pg/ml) for 30 minutes, and then incubated with MEV (200 μM) or GGPP (10 μM) for 24 hours or 8 hours, respectively, before harvest. Increased DNA binding activity of NF-κB in cells exposed to TNFα (blue bar) was observed, competed for by the wild-type consensus oligonucleotide (red bar) but not by the mutated oligonucleotide (yellow bar). Bars show the mean and SEM in samples from 5 RA patients in 1 of 5 separate experiments. **C,** Total cell lysates from rheumatoid synoviocytes treated with TNFα (100 pg/ml) in the presence or absence of SMV, MEV, and GGPP were analyzed by Western blotting for IκBα expression. A representative gel (upper panel) from 1 of 3 separate experiments is shown. Bars (lower panel) show the mean and SEM from 3 separate experiments. $* = P < 0.01$ versus control; $** = P < 0.05$ versus TNFα alone; **#** $= P < 0.01$ versus TNFα + SMV; **##** $= P < 0.05$ versus TNFα + SMV. OD = optical density (see Figure 1 for other definitions).

total RhoA protein levels. RhoA activity peaked following stimulation with TNFα at a concentration of 100 pg/ml, as compared with that in the medium-alone control (Figure 1A). Accordingly, the optimal concentration of TNFα was defined as 100 pg/ml for further experiments.

To determine the temporal profile of TNFα-induced RhoA activation, rheumatoid synoviocytes were stimulated with TNFα (100 pg/ml) for different periods of time (0, 5, 10, 20, and 30 minutes). As shown in Figure 1B, RhoA activity peaked at 10 minutes. In normal synoviocytes, elevated RhoA activity was also observed after TNFα stimulation, but the degree of RhoA activation by TNFα was significantly less pronounced than that in rheumatoid synoviocytes (Figure 1C).

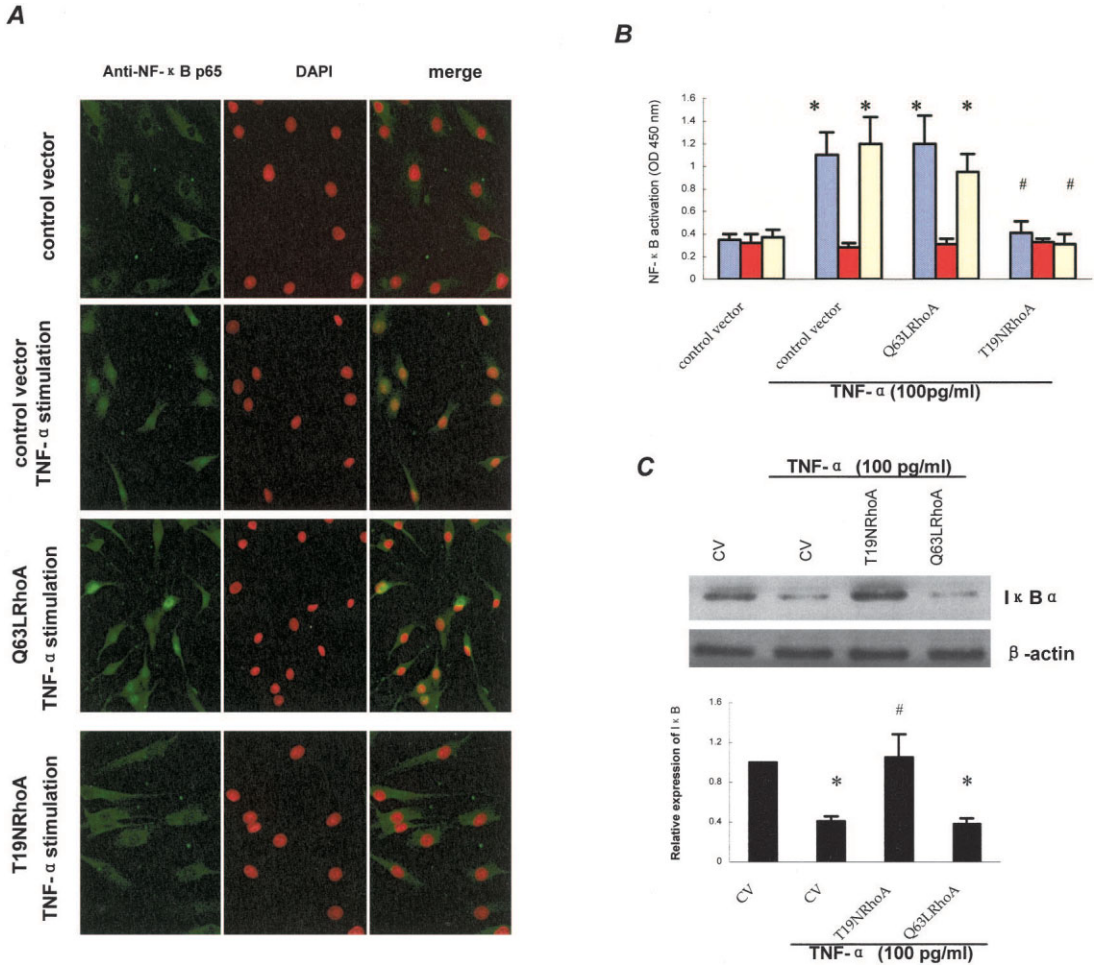

Figure 3. TNFα-induced NF-κB activation in rheumatoid synoviocytes mediated by RhoA. Rheumatoid synoviocytes were transfected with a dominant-negative mutant of RhoA (T19NRhoA), wild-type RhoA (Q63LRhoA), or control vector. **A,** Nuclear translocation of p65 was assessed by confocal fluorescence microscopy. Representative laser confocal microcopy images show nuclear translocation and colocalization of p65 (green stain) with nuclei stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (red stain) in dominant-negative RhoA– and wild-type RhoA–transfected cells exposed to TNFα (100 pg/ml). **B,** Decreased DNA binding capacity of NF-κB in dominant-negative RhoA–transfected cells exposed to TNFα was observed (blue bar) and effectively competed for by the wild-type consensus oligonucleotide (red bar) but not by the mutated oligonucleotide (yellow bar). Bars show the mean and SEM in samples from 5 RA patients in 1 of 5 separate experiments. $* = P < 0.01$ versus control vector without TNFα; **#** $= P < 0.05$ versus wild-type RhoA + TNFα. **C,** Effect of dominant-negative RhoA on IκBα degradation. A representative gel from 1 of 3 separate experiments is shown (upper panel). Bars show the mean and SEM in 3 RA samples from 1 of 3 separate experiments (lower panel). CV = control vector. $* = P < 0.05$ versus control vector without TNFα; **#** $= P < 0.05$ versus control vector + TNFα and versus wild-type RhoA + TNFα. OD = optical density (see Figure 1 for other definitions).

To study the effect of SMV on TNFα-induced RhoA activity, cells were pretreated with SMV (1 μM) for 18 hours before cotreatment with TNFα (100 pg/ml). As shown in Figure 1D, cotreatment with SMV prevented the TNFα-induced increase in RhoA activity. Total RhoA protein levels remained unchanged in response to SMV. Incubation of cells with SMV plus MEV (200 μM) or GGPP (10 μM) completely reversed the inhibitory effect of SMV on RhoA activation (Figure 1D).

Effect of TNFα and SMV on NF-κB activation. To examine whether NF-κB activation and the modulatory effect of SMV on TNFα-induced NF-κB activity are involved in the TNFα signaling pathway in RA, rheumatoid synoviocytes were incubated with TNFα (100 pg/ml) for 30 minutes in the presence or absence of SMV (1 μM). For studying the nuclear translocation of NF-κB, we performed laser scanning confocal immunofluorescence microcopy using anti-p65 antibody, a major subunit of NF-κB. As shown in Figure 2A, immunofluores-

cence staining revealed the translocation of p65 into the nucleus of rheumatoid synoviocytes following treatment with TNFα. Cotreatment of cells with SMV prevented TNFα-induced nuclear translocation of p65.

NF-κB DNA binding activity was also measured using a sensitive colorimetric assay, with a specific oligonucleotide probe for NF-κB. Rheumatoid synoviocytes treated with TNFα showed an increase in NF-κB DNA binding capacity (Figure 2B). Upon treatment with SMV (1 μM), the NF-κB binding capacity was decreased. Furthermore, MEV (200 μM) and GGPP (10 μM), which reversed the effect of SMV on RhoA activation, also completely reversed the effect of SMV on NF-κB DNA binding capacity.

Degradation of IκB plays a critical role in translocation of NF-κB into the nucleus. For studying the potential involvement of IκB in the TNFα-induced signaling pathway, we performed Western blot analysis using a specific anti-IκBα antibody. As shown in Figure 2C, rheumatoid synoviocytes stimulated with TNFα (100 pg/ml) exhibited decreased IκBα expression. SMV (1 μM) prevented the effect of TNFα on degradation of IκBα. Cotreatment of cells with MEV (200 μM) and GGPP (10 μM) completely reversed the inhibitory effect of SMV on IκBα.

Effect of dominant-negative RhoA mutant on NF-κB activation. Since recent studies indicated that RhoA is one of the major regulators of NF-κB activity (38), we further examined whether RhoA mediates TNFα-induced NF-κB activation in rheumatoid synoviocytes. To this end, the effects on nuclear translocation of p65 and NF-κB DNA binding activity were determined in cells transfected with a dominant-negative mutant of RhoA (T19NRhoA) or wild-type RhoA (Q63LRhoA). As shown in Figure 3A, cells transfected with the dominant-negative RhoA showed a significant decrease in TNFα-induced nuclear translocation of p65 and NF-κB DNA binding activity, indicating that TNFα-induced NF-κB activation is mediated by a RhoA-dependent pathway.

For establishing a sequential link between TNFα-induced RhoA activation and degradation of IκB, the expression of IκBα protein in cells transfected with dominant-negative RhoA and wild-type RhoA was determined by Western blotting. As shown in Figure 3C, TNFα stimulation failed to degradate IκBα protein in cells transfected with dominant-negative RhoA plasmid. These data indicate that RhoA is involved in TNFα-induced IκB degradation, which mediates the NF-κB signaling pathway.

Effect of TNFα and SMV on the NF-κB reporter gene. To investigate the role of TNFα and SMV on NF-κB gene transcription in rheumatoid synoviocytes, we transfected the cells with an NF-κB–dependent

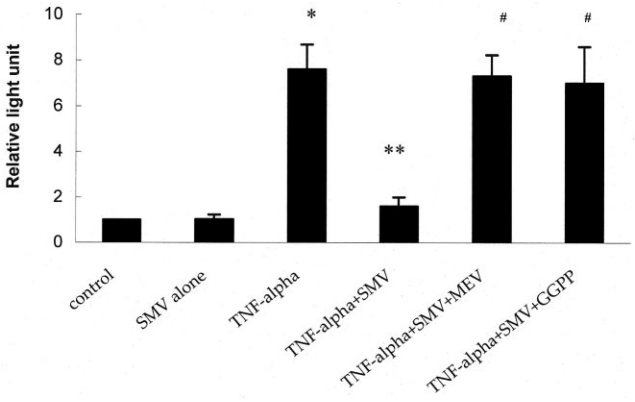

Figure 4. Effects of TNFα and SMV on NF-κB regulation of reporter gene expression. RA synoviocytes, transfected with an NF-κB–dependent luciferase gene reporter plasmid, were left untreated or were pretreated for 18 hours with SMV (1 μM), and then stimulated with TNFα (100 pg/ml) for 6 hours. MEV (200 μM) and GGPP (10 μM) were added for 24 hours or 8 hours, respectively, before harvest. The cells were harvested for detection of luciferase activity. Bars show the mean and SEM relative light units in samples from 3 RA patients in 1 of 3 separate experiments. $* = P < 0.05$ versus control; $** = P < 0.05$ versus TNFα; **#** $= P < 0.05$ versus TNFα + SMV. See Figure 1 for definitions.

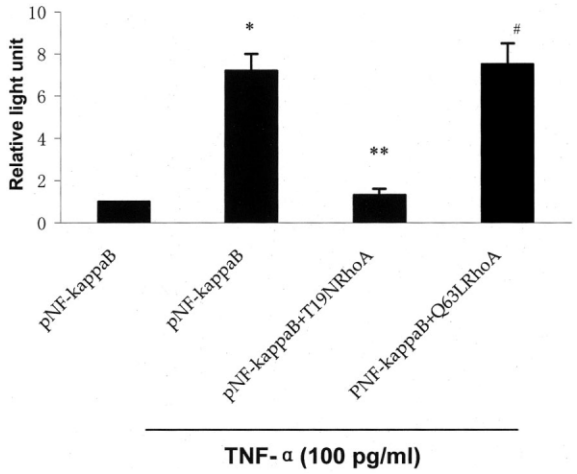

Figure 5. NF-κB regulation of reporter gene expression in TNFα-stimulated synoviocytes mediated by RhoA. The cells were cotransfected with an NF-κB–dependent luciferase gene reporter plasmid and/or dominant-negative RhoA (T19NRhoA) plasmid. The cells were stimulated with serum-free medium alone or TNFα (100 pg/ml) for 6 hours, and then lysed for detection of luciferase activity. Bars show the mean and SEM relative light units in samples from 3 RA patients in 1 of 3 independent experiments. $* = P < 0.05$ versus pNF-κB; $** = P < 0.05$ versus pNF-κB + TNFα; **#** $= P < 0.05$ versus pNF-κB + dominant-negative RhoA + TNFα. See Figure 1 for definitions.

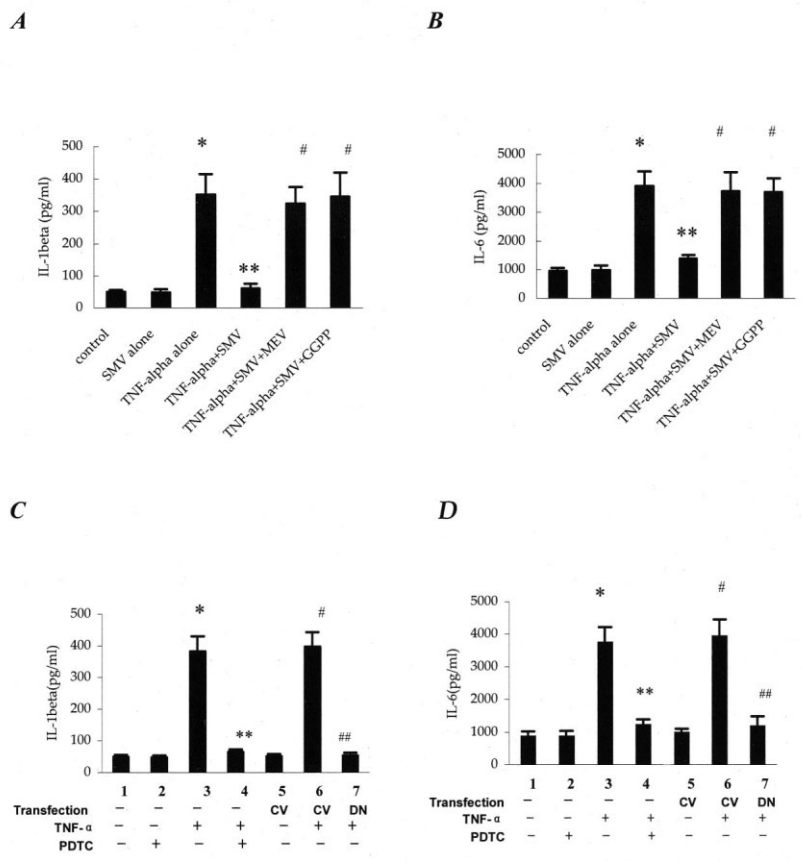

Figure 6. Induction of interleukin-1β (IL-1β) and IL-6 secretion by TNFα via RhoA-mediated NF-κB signaling in rheumatoid synoviocytes. **A** and **B,** Effect of TNFα and SMV on IL-1β **(A)** and IL-6 **(B)** secretion by rheumatoid synoviocytes. Cells, starved for 24 hours in RPMI 1640 containing 1% fetal calf serum, were left untreated or were pretreated for 18 hours with SMV (1 μM) and then stimulated with TNFα (100 pg/ml) for 12 hours. MEV (200 μM) and GGPP (10 μM) were added for 24 hours or 8 hours, respectively, before harvest. Bars show the mean and SEM in samples from 5 RA patients in 1 of 5 independent experiments. $* = P < 0.05$ versus control; $** = P < 0.05$ versus TNFα alone; $\# = P < 0.05$ versus TNFα + SMV. **C** and **D,** Effect of RhoA-mediated NF-κB signaling on TNFα-induced IL-1β **(C)** and IL-6 **(D)** secretion. Rheumatoid synoviocytes were transfected with control vector (CV) or dominant-negative (DN) RhoA plasmid (T19NRhoA). Cells were left untreated or were pretreated for 1 hour with pyrrolidine dithiocarbamate (PDTC) (300 μM) and then stimulated with TNFα (100 pg/ml) for 12 hours. Bars show the mean and SEM in samples from 5 independent donors in 1 of 5 independent experiments. $* = P < 0.05$ versus control; $** = P < 0.05$ versus TNFα alone in **A** and **B** and versus group 3 in **C** and **D**; $\# = P < 0.05$ versus TNFα + SMV in **A** and **B** and versus group 5 in **C** and **D**; $\#\# = P < 0.05$ versus group 6. See Figure 1 for other definitions.

luciferase gene reporter plasmid. As shown in Figure 4, TNFα stimulation caused a significant increase in NF-κB–dependent transcription of the luciferase reporter gene, and cotreatment of the cells with SMV reduced the TNFα-related NF-κB transcriptional activity. MEV (200 μM) and GGPP (10 μM) completely reversed the inhibitory effect of SMV on NF-κB transcriptional activity.

To confirm that the presence of RhoA is required in TNFα-induced NF-κB transcriptional activity, rheumatoid synoviocytes were cotransfected with an expression vector encoding a dominant-negative form of RhoA and an NF-κB reporter plasmid, before TNFα stimulation. Cotransfection of the dominant-negative RhoA plasmid with the NF-κB reporter plasmid decreased TNFα-stimulated luciferase activity (Figure 5). These results further confirm that RhoA mediates TNFα-induced NF-κB activation in rheumatoid synoviocytes.

Effect of TNFα and SMV on secretion of IL-1 and IL-6. The cytokines IL-1β and IL-6 are critical in the pathogenesis of RA. In this set of experiments, TNFα-stimulated secretion of IL-1β and IL-6 by rheumatoid synoviocytes was determined by ELISA. The cells were made quiescent by serum deprivation for 24 hours and then exposed to TNFα (100 pg/ml) for 12 hours. As shown in Figures 6A and B, TNFα stimulation caused a significant increase in the concentrations of IL-1β and IL-6 in supernatants of rheumatoid synoviocytes. Cotreatment of cells with SMV (1 μM) attenuated the TNFα-induced increase in IL-1β and IL-6 levels; however, MEV (200 μM) and GGPP (10 μM) completely reversed the inhibitory effect of SMV on secretion of IL-1β and IL-6.

To determine whether RhoA regulates TNFα-induced secretion of IL-1β and IL-6, rheumatoid synoviocytes were transfected with dominant-negative RhoA and stimulated with TNFα (100 pg/ml). As shown in Figures 6C and D, TNFα stimulation did not increase supernatant levels of IL-1β and IL-6 in the cells transfected with dominant-negative RhoA, indicating that TNFα-induced IL-1β and IL-6 secretion is mediated by a RhoA-dependent pathway. In addition, we also demonstrated that PDTC, an inhibitor of NF-κB, markedly reduced the increases in IL-1β and IL-6 levels that were induced by TNFα, suggesting that the effects of NF-κB on TNFα-stimulated cytokine secretion are modulated by rheumatoid synoviocytes.

DISCUSSION

The present study in cultured rheumatoid synoviocytes shows that TNFα-induced NF-κB activation is dependent on the activity of RhoA. Our results also provide evidence that SMV inhibits TNFα-induced activation of NF-κB and secretion of IL-1β and IL-6 in rheumatoid synoviocytes by preventing signaling in the RhoA pathway.

A great deal of evidence indicates that in activated rheumatoid synoviocytes, many pathologic processes, including production of inflammatory cytokines, are regulated by intracellular signaling. The Rho family of small GTPases contains important regulators that are involved in a number of intracellular signaling pathways, such as actin stress fiber formation, cell proliferation, and transcriptional regulation (15,16,39). Although it has been reported that thrombin can induce proliferation, progression of the cell cycle to the S phase, and IL-6 secretion by RA synovial fibroblasts through Rho and one of its guanine nucleotide exchange factors (GEFs), p115RhoGEF (21), the role of Rho-mediated signaling in inflammatory processes in RA is still unknown.

Therefore, in the present study using cultured human rheumatoid fibroblast-like synoviocytes, we elucidated the pivotal role of RhoA in TNFα-induced activation of the NF-κB pathway, a critical signaling pathway for regulating the inflammatory response. We demonstrated that in rheumatoid synoviocytes, TNFα stimulation induced a dose-dependent increase in RhoA activity, suggesting that RhoA may play a role downstream in TNFα-stimulated signaling. We also showed that TNFα induced nuclear NF-κB translocation, DNA binding, and gene transcription, and further demonstrated that these effects were mediated by RhoA, since rheumatoid synoviocytes transfected with dominant-negative RhoA mutant failed to exhibit increased nuclear NF-κB translocation, DNA binding activity, and gene transcription in response to TNFα. This finding was consistent with the observed inhibition of degradation of IκB, a protein kinase that inhibits NF-κB activity, in the cells infected with dominant-negative RhoA plasmid. These results are identical to the findings in recent studies in which inactivation of the Rho protein could reduce TNFα-stimulated NF-κB activity in other cell lines (22,38,40,41).

IL-1β and IL-6 are critical cytokines in the pathogenesis of RA. These cytokines exhibit abundant production in RA synovium and high concentrations in the synovia and serum of patients with RA. Previous studies indicate that IL-1β can be induced via the RhoA-mediated NF-κB pathway in PBMCs from patients with Crohn's disease (22). Moreover, thrombin-induced IL-6 secretion is mediated by the RhoA pathway in rheumatoid synoviocytes (21). In this study, the obtained results showed that the TNFα-induced secretion of IL-1β and IL-6 was inhibited in rheumatoid synoviocytes expressing the dominant-negative mutant of RhoA, suggesting that RhoA plays a role in mediating the secretion of IL-1β and IL-6 by rheumatoid synoviocytes. We also found that PDTC, a specific inhibitor of NF-κB, markedly reduced supernatant levels of IL-1β and IL-6 following stimulation with TNFα. Taken together, these data indicate that RhoA plays a key role in synovial NF-κB activation and cytokine secretion in the TNFα-stimulated process of synovial inflammation.

Because the role of NF-κB activation in RA is well documented, inhibition of cytoplasmic components of NF-κB, such as RhoA, may be an effective strategy for blocking the inflammatory process. Our study findings suggest that specific inhibition of RhoA activation may be considered a promising antiinflammatory approach with therapeutic potential in RA.

Statins are drugs that are commonly prescribed for the treatment of patients with hypercholesterolemia and have been suggested to exert an antiinflammatory role by lipid-lowering–independent functions. Although it has been reported that SMV is beneficial for inflammatory arthritis (30), the exact mechanisms by which statins modulate the RA inflammatory response are still unknown in detail. Recent reports indicate that statins can influence the signaling pathways implicated in the modulation of inflammatory processes in several cell lines (25). For instance, Hernández-Presa et al reported that SMV prevented NF-κB activation in PBMCs (42). Meroni et al also demonstrated that statins could inhibit antiphospholipid antibody– and TNFα-induced activation of NF-κB and cytokine expression in endothelial cells (43). In the present study, we demonstrated that SMV inhibited TNFα-induced nuclear NF-κB translocation, DNA binding, and luciferase reporter gene expression in cultured rheumatoid synoviocytes, and further showed that SMV prevented degradation of IκBα, suggesting that SMV can modulate NF-κB activity and its related gene transcription in rheumatoid synoviocytes.

Activation of the NF-κB transcription factor family plays a central role in inflammatory responses through the ability of NF-κB to regulate proinflammatory gene transcription. Moreover, excessive NF-κB activation has been implicated in diverse chronic diseases, including RA (12,44). Inhibition of NF-κB activity has been shown to have antiinflammatory effects, both in cultured cells and in animal models of inflammatory arthritis (6–12). Thus, SMV may be effective by inducing an antiinflammatory response in RA through the modulation of inflammatory gene activation, via inhibition of NF-κB activity.

Increasing evidence suggests that statins exhibit significant pleiotropic effects on cell signaling pathways, largely by preventing posttranslational lipid modification (isoprenylation) of small GTPase proteins, a process essential for the translocation of Rho GTPases from the cytosol to the membrane, where activation of these proteins takes place, and which influences numerous inflammatory signaling pathways (45–49). Previous studies from our laboratory and other investigators have indicated that statins modulate several cellular processes by preventing prenylation of small Rho GTPases such as Rho and Rac1 GTPases (34,35).

In the present study, we found that cotreatment of rheumatoid synoviocytes with SMV prevented the activation of RhoA induced by TNFα, and at the same concentrations as those that were found to inhibit NF-κB activation, luciferase reporter gene expression, and cytokine secretion. Therefore, our results suggest that SMV plays a beneficial role in inflammatory arthritis by, at least in part, preventing RhoA-mediated NF-κB signaling and inhibition of proinflammatory cytokine secretion. Furthermore, MEV and GGPP not only reversed the inhibitory effect of SMV on RhoA activation, but also reversed the SMV inhibition of NF-κB activation and proinflammatory cytokine secretion by rheumatoid synoviocytes. These data suggest that SMV interfered with TNFα-induced NF-κB activation in rheumatoid synoviocytes via inhibition of geranylgeranylation of Rho.

In conclusion, on the basis of our findings, we propose that RhoA is involved in TNFα-induced NF-κB activation and cytokine secretion, suggesting a key role of the Rho GTPase in inflammatory responses and arthritic inflammation. Our study also demonstrates, for the first time, that SMV, by preventing RhoA activity, modulates TNFα-induced NF-κB activation and proinflammatory cytokine secretion in RA.

ACKNOWLEDGMENTS

We thank Ning Luo and Yajie Zhang for technical assistance, and Dr. Weiyi Mai for his assistance in manuscript preparation.

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