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*J Immunol* 2007; 178:2507-2516; doi: 10.4049/jimmunol.178.4.2507

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Simvastatin Potentiates TNF-α-Induced Apoptosis through the Down-Regulation of NF-κB-Dependent Antiapoptotic Gene Products: Role of IκBα Kinase and TGF-β-Activated Kinase-1

Kwang Seok Ahn, Gautam Sethi, and Bharat B. Aggarwal

Numerous recent reports suggest that statins (hydroxy-3-methylglutaryl-CoA reductase inhibitors) exhibit potential to suppress tumorigenesis through a mechanism that is not fully understood. Therefore, in this article, we investigated the effects of simvastatin on TNF-α-induced cell signaling. We found that simvastatin potentiated the apoptosis induced by TNF-α as indicated by intracellular esterase activity, caspase activation, TUNEL, and annexin V staining. This effect of simvastatin correlated with down-regulation of various gene products that mediate cell proliferation (cyclooxygenase-2), vascular endothelial growth factor (VEGF), and angiogenesis (vascular endothelial growth factor); all known to be regulated by the NF-κB pathway. We found that simvastatin inhibited NF-κB activation, and 1-imevalonate reversed the suppressive effect, indicating the role of hydroxy-3-methylglutaryl-CoA reductase. Simvastatin suppressed not only the inducible but also the constitutive NF-κB activation. Simvastatin inhibited TNF-α-induced IκBα kinase activation, which led to inhibition of IκBα phosphorylation and degradation, suppression of p65 phosphorylation, and translocation to the nucleus. NF-κB-dependent reporter gene expression induced by TNF-α, TNFR1, TNFR-associated death domain protein, TNFR-associated factor 2, TGF-β-activated kinase 1, receptor-interacting protein, NF-κB-inducing kinase, and IκB kinase β was abolished by simvastatin. Overall, our results provide novel insight into the role of simvastatin in potentially preventing and treating cancer through modulation of IκB kinase and NF-κB-regulated gene products. The Journal of Immunology, 2007, 178: 2507-2516.

Perhaps the potential of folk medicine for the treatment of human disease cannot be better illustrated than by that of the story of statins. Red yeast rice, prepared by adding purple-colored yeast (Monascus purpureus) to steamed rice, has been consumed in China for thousands of years to improve digestion and blood circulation. Among its many constituents, this fermented mix contains molecules called statins, which have a high affinity for 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, a rate-limiting enzyme in cholesterol biosynthesis pathway (1–4). The statins derived from red yeast rice fermentation include lovastatin, mevatatin, simvastatin (a methyl derivative of lovastatin), and pravastatin; whereas fluvastatin, atorvastatin, cerivastatin, rosuvastatin, and pitavastatin are synthetic compounds. Besides cholesterol-lowering activity, there is increasing evidence that statins have potential in a wide variety of diseases, including cancer (5–7). Statins have also been shown to promote bone formation (8), modulate immune system (9), suppress inflammation (10), modulate angiogenesis (11, 12), and inhibit the proliferation of wide variety of cells (5–7, 13–15).

Besides inhibiting HMG-CoA reductase, statins appear to mediate their effects by targeting various other cell signaling pathways. Statins have been shown to bind lymphocyte function-associated Ag-1 (16), inhibit geranylgeranylation of p protein (17), regulate cyclin-dependent kinase inhibitors p21 (18) and p27 (14), and interfere with the Raf/mitogen-activated protein extracellular cysteine-rich kinase/extracellular signal-regulated protein kinase pathway (15). The use of lovastatin in the synchronization of cells at the G1 phase of the cell cycle has been well described (19).

Because of the role of the transcription factor NF-κB in tumorigenesis, apoptosis, inflammation, immunomodulation, angiogenesis, and bone remodeling (20), we speculated that statins mediate their effects by modulating the NF-κB pathway. Our results demonstrate that simvastatin potentiates the TNF-α-induced apoptosis through down-regulation of NF-κB regulated antiapoptotic gene products and the NF-κB activation pathway.

Materials and Methods

Materials

A solution of 100% DMSO and 50 mM simvastatin (LKT Laboratories) was prepared and stored as small aliquots at −20°C and then diluted as needed in cell culture medium. Bacteria-derived recombinant human TNF-α, purified to homogeneity with a specific activity of 5 × 10⁷ U/mg,
was provided by Genentech. Penicillin, streptomycin, IMDM, and FBS were obtained from Invitrogen Life Technologies. Anti-β-actin and anti-FLAG Abs were obtained from Sigma-Aldrich. Abs against p65, p50, IκBα, cyclin D1, matrix metalloproteinase-9 (MMP-9), poly(ADP-ribose) polymerase (PARP), inhibitor of apoptosis protein (IAP)1, IAP2, Bcl-2, Bcl-xL, ICAM-1, and the annexin V staining kit were obtained from Santa Cruz Biotechnology. Vascular endothelial cell growth factor (VEGF) Ab was purchased from Neomarkers. Anti-cyclooxygenase-2 (COX-2) Ab was obtained from BD Biosciences. Phospho-specific anti-IκBα (Ser276/286) and phospho-specific anti-p65 (Ser536) were purchased from Cell Signaling Technology. Anti-IκBα kinase (IKK)-α, anti-cellular FLIP (cFLIP), and anti-IKK-β Abs were provided by Ingenex. Expressions vectors plasmids for TGFβ-activated kinase (TAK1), TAK1-binding protein (TAB1), and receptor-interacting protein (RIP) were provided by Dr. X. Lin (University of Texas, M. D. Anderson Cancer Center, Houston, TX) (21).

Cell lines

Human myeloid KBM-5, squamous cell carcinoma SCC4, and human embryonic kidney A293 cells were obtained from American Type Culture Collection. KBM-5 cells were cultured in IMDM supplemented with 15% FBS. A293 cells were cultured in DMEM supplemented with 10% FBS. SCC4 cells were cultured in DMEM containing 10% FBS, nonessential amino acids, pyruvate, glutamine, and vitamins. All media were also supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin.

Live/Dead assay

To measure apoptosis, we used the Live and Dead assay (Molecular Probes), which determines intracellular esterase activity and plasma membrane integrity. This assay uses calcein, a polyanionic dye, which is retained in live cells and provides green fluorescence (22). It also uses the ethidium monomer dye (red fluorescence), which can enter cells only through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membrane of live cells.

In brief, 10^5 cells were incubated with 50 µM simvastatin for 2 h and then treated with 1.0 mM TNF-α for 24 h at 37°C. Cells were stained with the Live and Dead reagent (5 µM ethidium homodimer and 5 µM calcein-AM) and then incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon).

Annexin V assay

One of the early indicators of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylinerine from the cell’s cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected using the binding properties of annexin V. To detect apoptosis, we used annexin V Ab conjugated with the fluorescent dye FITC. In brief, 1 × 10^6 cells were pretreated with 50 µM simvastatin for 12 h, treated with 1.0 nM TNF-α for 24 h, and subjected to annexin V staining. Cells were washed, stained with FITC-conjugated anti-annexin V Ab, and analyzed with a flow cytometer (FACSCalibur; BD Biosciences).

TUNEL

We also assayed apoptosis by the TUNEL method, which is used to examine DNA strand breaks during apoptosis with an in situ cell death detection reagent (Roche Molecular Biochemicals). In brief, 5 × 10^5 cells were incubated with 50 µM simvastatin for 12 h and then treated with 1.0 nM TNF-α for 24 h at 37°C. Thereafter, cells were washed, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After being washed, cells were incubated with reaction mixture for 30 min at 37°C. Stained cells were analyzed with a flow cytometer (FACSCalibur; BD Biosciences).

PARP cleavage assay

To detect cleavage products of PARP, whole-cell extracts were prepared by subjecting simvastatin-treated cells to lysis in lysis buffer (20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 µg/ml aprotinin, 0.005 µg/ml leupeptin, 0.4 mM PMSF, and 4 mM NaVO₃). Lysates were spun at 14,000 rpm for 10 min to remove insoluble material, resolved by 10% SDS-PAGE, and probed with PARP Ab.

Western blot analysis

To determine the effect of simvastatin on TNF-α-dependent IκBα phosphorylation, IκBα degradation, p65 translocation, and p65 phosphorylation, cytoplasmic extracts were prepared as previously described (23) from KRM-5 cells (2 × 10⁶/ml) that had been pretreated with 50 µM simvastatin for 12 h and then exposed to 0.1 nM TNF-α for indicated times. Cytoplasmic protein (30 µg) was resolved on 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with specific Abs against IκBα, phosphorylated IκBα, p65, and phosphorylated p65. Next, to determine the expression of cyclin D1, COX-2, MMP-9, IAP1, IAP2, Bcl-2, Bcl-xL, VEGF, ICAM-1, cFLIP, and survivin in whole-cell extracts of treated cells (2 × 10⁶ cells/ml), 50 µg of whole-cell lysate was resolved by SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then probed with Abs against various proteins. The blots were washed, exposed to HRP-conjugated secondary Abs for 1 h, and detected by ECL reagent (Amersham Biosciences). The bands were quantified using a personal densitometer scan version 1.30 and ImageQuant software version 3.3 (Molecular Dynamics).

EMSA

We performed the EMSA essentially as described previously (24). In brief, nuclear extracts prepared from TNF-α-treated cells (2 × 10⁶/ml) were incubated with 32P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 µg of protein with 16 fmol DNA) from the HIV long terminal repeat 5'-TTTTTACCAAGGACCTTCCGGCGTGGGTTTCCAGGAGGGGCGG-3' (boldface indicates NF-κB binding sites) for 30 min at 37°C, and the DNA-protein complex formed was separated from free oligonucleotides on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5'-TTTCCACAGCTTCCATTCCCTTGGGACTTTCCAGGAGGGGCGG-3' (boldface indicates NF-κB binding sites), was used to determine the specificity of NF-κB binding to DNA. The specificity of binding was also evaluated by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-α-treated cells were incubated with Abs against the p50 or p65 subunit of NF-κB for 30 min at 37°C before the complex was analyzed by EMSA. Preimmune serum (PIS) was included as a negative control. The dried gels were visualized with a Storm 820, and radioactive bands were quantified using ImageQuant software (Amersham Biosciences).

IKK assay

To determine the effect of simvastatin on TNF-α-induced IKK activation, we analyzed IKK activation essentially as described previously (23). In brief, the IKK complex from whole-cell extracts was precipitated overnight with Ab against IKKα and IKKβ and then treated with protein A/G-agarose beads ( Pierce). After 2 h, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM MgCl₂, 2 mM DTT, 20 µCi of [γ-32P]ATP, 10 µM unlabeled ATP, and 2 µg of substrate GST-IκBα (aa 1–54). After being incubated at 30°C for 30 min, the reaction was terminated by being boiled with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE. The gel was dried, and the radiactive bands were visualized with a Storm 820 PhosphorImager. To determine the total amounts of IKKα and IKKβ in each sample, 50 µg of whole-cell proteins were resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKKα or anti-IKK-β Ab.

NF-κB-dependent reporter gene transcription

The effect of simvastatin on TNF-α-induced NF-κB-dependent reporter gene transcription in A293 cells was measured as described previously (23). For TAK1/TAB1 and RIP experiments, A293 cells (5 × 10⁶ cells/well) were transiently transfected with the expression vectors for TAK1/TAB1 or RIP and pNF-κB-secretory alkaline phosphatase (SEAP) (0.5 µg) plasmids by the FuGene6 method (Roche Molecular Biochemicals). After 24 h, cells were treated with indicated concentrations of simvastatin, and conditioned medium was harvested after 12 h for SEAP activity. Cells were cotransfected with β-galactosidase and normalized the data with β-galactosidase assay. Also, transfected cells that overexpress FLAG-TAK1 protein were probed with specific Ab against FLAG (data not shown).

COX-2 promoter-dependent reporter luciferase gene expression

COX-2 promoter activity was evaluated as described elsewhere (23). To further determine the effect of simvastatin on COX-2 promoter, we seeded A293 cells at a concentration of 1.5 × 10⁵ cells/well in 6-well plates. After being cultured overnight, the cells in each well were transfected with 2 µg of DNA consisting of COX-2 promoter-luciferase reporter plasmid with 6 µl of LipofectAMINE 2000 (Invitrogen Life Technologies) according to the manufacturer’s protocol. The COX-2 promoter (~375 to +59), which was amplified from human genomic DNA using the primers 5’-GAGTCTTC TTATTTATTTTT-3' (sense) and 5’-GCTGCGAGGAATCCGGAC GTGC-3' (antisense), was provided by Dr. X.-C. Xu (University of Texas Southwestern Medical Center). The transfected cells were incubated for 12 h, and then 0.1 nM TNF-α was added to each well, and then cultured for 24 h. The luciferase activity was assayed using a luminometer (Wallac 1420).
M. D. Anderson Cancer Center, Houston, TX). After a 6-h exposure to the transfection mixture, the cells were incubated in medium containing simvastatin for 12 h. The cells were exposed to TNF-α (1 nM) for 24 h and harvested. Luciferase activity was measured using the Lumicite luciferase assay system (PerkinElmer Life and Analytical Sciences) according to the manufacturer’s protocol and detected with a luminometer (Victor 3; PerkinElmer Life and Analytical Sciences). All experiments were performed in triplicate and repeated at least twice to establish their reproducibility.

Results
This study was designed to investigate the effects of simvastatin on the TNF-α-induced NF-κB activation pathway, NF-κB-regulated gene expression, and on apoptosis. Simvastatin, derived from natural source, was used to investigate TNF-α signaling. Most of the studies were conducted using human chronic myeloid leukemia (KBM-5) cells because these cells express both types of TNFRs.

Simvastatin enhances the apoptotic effects of TNF-α
NF-κB activation inhibits apoptosis induced by TNF-α (25). So we determined the potential of simvastatin to enhance apoptosis induced by TNF-α using the Live and Dead, PARP cleavage, annexin V staining, and TUNEL staining methods. The Live and Dead assay, which measures intracellular esterase activity and plasma membrane integrity, indicated that simvastatin up-regulated TNF-α-induced apoptosis, from 5 to 50% (Fig. 1A). Similarly, annexin V staining also showed that simvastatin is effective at enhancing the effects of TNF-α (Fig. 1B). TUNEL staining indicated that apoptosis was enhanced by incubation with simvastatin (Fig. 1C). By using caspase-activated PARP cleavage, we also showed that simvastatin enhanced TNF-α-induced apoptosis (Fig. 1D). The results of all these assays suggest that simvastatin enhances the apoptotic effects of TNF-α. TNF-α alone was minimally effective in inducing apoptosis in all these assays because suboptimal doses of TNF-α were used.

Simvastatin inhibits TNFα-induced expression of proliferation gene products
Both cyclin D1 and COX-2 contribute to carcinogenesis by promoting cell proliferation. Cyclin D1 is a protein that controls the transition from G1-S phase in the cell cycle and is overexpressed in a variety of tumors (26). COX-2 is an enzyme that catalyzes the production of PGE2 from arachidonic acid. The production of PGE2 has been linked to proliferation and metastasis of tumor cells (27). Therefore, we investigated whether simvastatin could modulate the expression of COX-2 and cyclin D1 gene products. We found that TNF-α treatment induced the expression of both these proteins and that simvastatin diminished the expression (Fig. 2A).

Simvastatin inhibits TNFα-induced expression of antiapoptotic gene products
We found that simvastatin potentiated the apoptotic effects of TNF-α. Because anti-apoptotic proteins such as, IAP1, IAP2, Bcl-2, Bcl-xL, cFLIP, and survivin play a major role in evading apoptosis and prolonging survival of cancer cells (25), we examined whether simvastatin can modulate the expression of these anti-apoptotic gene products induced by TNF-α in KBM-5 cells. The results of Western blot analysis showed that TNF-α induced the expression of these anti-apoptotic proteins in a time-dependent manner and that simvastatin suppressed it (Fig. 2B). cFLIP inhibits death receptor-induced apoptosis by binding to FADD (Fas-associated death domain protein) and procaspase-8 (28). Survivin, an IAP, localizes to the intermitochondrial membrane space in tumor cells and this localization accelerates tumorigenesis (29). Interestingly we observed that simvastatin completely down-regulated TNF-α-induced expression of survivin and cFLIP proteins. The suppression of expression of some gene products was more pronounced (such as cFLIP and survivin) than others (such as bcl-2, bcl-xl, and IAP1). This difference could be due to either variation in basal expression or complex regulation.

Simvastatin inhibits TNFα-induced expression of angiogenic and invasion-associated gene products
VEGF plays a critical role in angiogenesis by promoting the growth of vascular endothelial cells and enhancing vascular permeability (30), whereas MMP-9 by virtue of its property to degrade extracellular matrix has been implicated in cellular invasion
ICAM-1, an adhesion molecule has also been shown to be required for metastasis of tumors (32). Hence, we determined whether simvastatin could modulate TNF-α-induced expression of VEGF, MMP-9, and ICAM-1 proteins. We found that TNF-α treatment induced the expression of VEGF, MMP-9, and ICAM-1 gene products in a time-dependent manner and that simvastatin inhibited the expression (Fig. 2). Simvastatin inhibits TNF-α-induced NF-κB activation in a dose-dependent manner

Our results till now show that simvastatin potentiates TNF-α-induced apoptosis and down-regulates various gene products involved in cell proliferation, survival, angiogenesis and invasion, all known to be regulated by NF-κB activation (33). We next determined whether simvastatin affects TNF-α-induced NF-κB activation. KBM-5 cells were pretreated with different doses of simvastatin for 12 h and then treated with 1 nM TNF-α for 30 min. Nuclear extracts were prepared and then tested for NF-κB activation as described in Materials and Methods. A, Simvastatin inhibits TNF-α-dependent NF-κB activation. KBM-5 cells (2 x 10⁶/ml) were preincubated with 50 μM simvastatin for the indicated times at 37°C and then treated with 0.1 nM TNF-α for 30 min at 37°C. Nuclear extracts were prepared and then tested for NF-κB activation. C, NF-κB induced by TNF-α is composed of p65 and p50 subunits. Nuclear extracts from untreated or TNF-α-treated cells were incubated with the indicated Abs, PIS, unlabeled NF-κB oligo-probe, or mutant oligo-probe and then assayed for NF-κB activation by EMSA.

Simvastatin suppresses NF-κB activation in a time-dependent manner

We also investigated the length of incubation required for simvastatin to suppress TNF-α-induced NF-κB activation. KBM-5 cells were preincubated with the indicated concentrations of simvastatin for 12 h at 37°C and then treated with 0.1 nM TNF-α for 30 min. Nuclear extracts were prepared and tested for NF-κB activation as described in Materials and Methods. B, Simvastatin inhibits TNF-α-dependent NF-κB activation. KBM-5 cells (2 x 10⁶/ml) were preincubated with 50 μM simvastatin for the indicated times at 37°C and then treated with 0.1 nM TNF-α for 30 min at 37°C. Nuclear extracts were prepared and then tested for NF-κB activation.
Simvastatin does not interfere with the binding of NF-κB to DNA

Several agents have been described that suppress NF-κB binding to the DNA, e.g., caffeic acid phenethyl ester (37), herbimycin (38), plumbagin (39), ethyl caffeate (40), and tosyl-l-phenylalanyl-

molecular mass (Fig. 3). Simvastatin inhibits TNF-α-induced IKK activation.

Simvastatin inhibits TNFα-dependent IkBα degradation

The translocation of NF-κB to the nucleus is preceded by the proteolytic degradation of IkBα (20). To determine whether NF-κB inhibitory activity of simvastatin was due to inhibition of IkBα degradation, we pretreated cells with simvastatin, exposed them to 0.1 nM TNF-α for different times, and examined the IkBα status in the cytoplasm by Western blot analysis. TNF-α induced IkBα degradation in control cells within 10 min and reached maximum at 15 min, but TNF-α could not induce on IkBα degradation in simvastatin pretreated cells (Fig. 5A). These results indicate that simvastatin inhibits TNF-α-induced IkBα degradation.

Simvastatin inhibits TNFα-induced IkBα phosphorylation

The proteolytic degradation of IkBα is known to require phosphorylation at serine residues 32 and 36 (42). To determine whether the inhibition of TNF-α-induced IkBα degradation was due to inhibition of IkBα phosphorylation, we used the proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal to block degradation of IkBα (43). KBM-5 cells were pretreated with simvastatin, treated with 50 μg/ml N-acetyl-leucyl-leucyl-norleucinal for 30 min, exposed to TNF-α, and then examined for IkBα phosphorylation status by Western blot analysis using an Ab that recognizes the serine-phosphorylated form of IkBα. TNF-α-induced phosphorylation of IkBα was undetectable, however, when cells were pretreated with the inhibitor, TNF-α-induced phosphorylation of IkBα was noted (Fig. 5B). Simvastatin significantly suppressed the IkBα phosphorylation induced by TNF-α in the presence of the proteasome inhibitor.

Simvastatin inhibits TNFα-induced IKK activation

Some agents activate NF-κB through an IKK-independent mechanism (44). However, IKK activation is required for TNF-α-induced phosphorylation of IkBα (34). Because simvastatin inhibits the phosphorylation of IkBα, we determined the effect of simvastatin on TNF-α-induced IKK activation. The results from the immune complex kinase assay showed that TNF-α activated IKK as early as 5 min after TNF-α treatment but that simvastatin strongly suppressed this activation (Fig. 5C). Simvastatin alone or in combination with TNF-α did not affect the expression of IKKa and IKKβ protein. These results suggest that simvastatin did not affect global protein synthesis of the cell.
Simvastatin inhibits TNFα-induced p65 translocation and phosphorylation

TNF-α induces the phosphorylation of p65, which is required for its transcriptional activity (42). Whether simvastatin modulates TNF-α-induced phosphorylation of p65 was examined. Western blot analysis showed that TNF-α induced the phosphorylation of p65 within 15 min and that simvastatin strongly suppressed this phosphorylation (Fig. 5D).

After phosphorylation, the NF-κB p65 subunit is translocated to the nucleus. Western blot analysis showed that TNF-α induced nuclear translocation of p65 in a time-dependent manner in KBM-5 cells, and pretreatment with simvastatin inhibited it (Fig. 5E).

Simvastatin represses TNFα-induced NF-κB-dependent reporter gene expression

Although we determined by EMSA that simvastatin inhibited NF-κB activation, DNA binding alone is not always associated with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps (45, 46). We investigated where simvastatin acts in the sequence of TNFR1, TRADD, TRAF2, NF-κB-inducing kinase (NIK), and IKK recruitment that characterizes TNF-α-induced NF-κB activation (47, 48). In cells transfected with TNFR1, TRADD, TRAF2, NIK, IKKβ, and p65 plasmids, NF-κB-dependent SEAP expression was induced; simvastatin substantially suppressed NF-κB-dependent SEAP expression in all cells except those transfected with p65 (Fig. 6A). Because IKK activation can cause the phosphorylation of IκBα and p65, we propose that simvastatin inhibits NF-κB activation through inhibition of IKK activation.

Simvastatin inhibits TAK1/TAB1-induced NF-κB-dependent reporter gene expression

TAK1, a member of the MAPKKK, was originally identified as a key regulator of MAPK activation in TGF-β-induced signaling pathways. It is activated by various inflammatory stimuli, including TNF-α, IL-1, and LPS (49). Recent studies indicate that TAK1 plays a major role in TNF-α-induced NF-κB activation through its interaction with TAB1 and TAB2 (50). Therefore, we investigated whether simvastatin suppresses TNF-α-induced NF-κB activation through the inhibition of TAK1. As shown in Fig. 6B, in cells transfected with TAK1/TAB1 NF-κB-dependent reporter gene expression was induced, and simvastatin treatment inhibited this activation in a dose-dependent manner.

Simvastatin inhibits RIP-induced NF-κB-dependent reporter gene expression

RIP is a serine/threonine kinase that plays an important role in TNF-α-induced NF-κB activation (51, 52). RIP is ubiquitinated following TNF-α stimulation, which has been reported to play an important role in NF-κB activation (53). Therefore, we investigated whether simvastatin suppresses TNF-α-induced NF-κB activation through the inhibition of RIP. As shown in Fig. 6C, RIP activated NF-κB reporter activity and simvastatin inhibited the activation in a dose-dependent manner.

Simvastatin represses TNFα-induced COX-2 promoter activity

We next determined whether simvastatin affected COX-2 promoter activity, which is regulated by NF-κB (54). As shown in Fig. 6D,
TNF-α induced COX-2 promoter activity, and simvastatin substantially reduced TNF-α-induced COX-2 promoter activity in a dose-dependent manner.

**Discussion**
This study was designed to investigate the effects of simvastatin on TNF-α-induced NF-κB activation pathway, NF-κB regulated gene expression and on NF-κB mediated cellular responses. We found that simvastatin potentiated the apoptosis induced by TNF-α. This effect of simvastatin correlated with down-regulation of various gene products that mediate cell proliferation, cell survival, invasion, and angiogenesis; all known to be regulated by NF-κB. We found that the simvastatin inhibited TNF-α-induced NF-κB activation, and mevalonate reversed its suppressive effect. Simvastatin
suppressed not only the inducible but also the constitutive NF-κB activation. Suppression of NF-κB activation by simvastatin is due to inhibition of TNF-α-induced TAK1 and IκB kinase activation, which led to inhibition of IκBα phosphorylation and degradation, suppression of p65 phosphorylation, and translocation to the nucleus; and inhibition of NF-κB-dependent reporter gene expression.

We found for the first time that simvastatin potentiates TNF-α-induced apoptosis in myeloid leukemia cells. When investigated for the mechanism of this potentiation, we found the down-regulation of various antiapoptotic gene products, such as Bcl-2, Bcl-xL, cFLIP, IAP1, IAP2, and survivin by simvastatin. The down-regulation of Bcl-2 by lovastatin has been reported previously (55). Like simvastatin, cycloheximide is also known to potentiate TNF-α-induced apoptosis (56); however, cycloheximide mediates its effects through suppression of protein synthesis, whereas simvastatin mediates its effects through suppression of NF-κB-regulated gene products as shown here. We found that simvastatin (50 μM for 12h) had no effect on protein synthesis as indicated by the expression of β-actin, PARP, IκKα, and IκKβ (Fig. 5). The antiproliferative effects of simvastatin, previously reported against a wide variety of tumor cells (6, 7, 14, 15), could be due to down-regulation of these gene products. We also found for the first time that expression of cyclin D1 and COX-2 was down-modulated by simvastatin. Indeed, lovastatin is used routinely to synchronize cells in the G1 phase (19), which could be due to down-regulation of cyclin D1, as shown here.

We also found that the expression of VEGF, MMP-9, and ICAM-1, the gene products that have been implicated in angiogenesis and invasion, were also suppressed by simvastatin. Our results are consistent with the report that simvastatin can suppress angiogenesis (12).

How simvastatin down-regulates the expression of such a wide variety of gene products involved in tumorigenesis, was investigated. We found for the first time that simvastatin is a potent inhibitor of TNF-α-induced NF-κB activation. We also found for the first time that simvastatin inhibited not only inducible NF-κB activation but also constitutively active NF-κB expressed by certain tumor cells. Our results are in agreement with a report that shows that statins can suppress LPS-induced NF-κB activation (57). We found for the first time that the NF-κB inhibitory activity of the simvastatin could be reversed by mevalonate, indicating that the HMG-CoA reductase pathway also has a role in NF-κB activation. Our results are in agreement with those of Holschermann et al. (58), who examined the effect of cerivastatin on TNF-α-induced NF-κB activation in endothelial cells. Mevalonate is not only a precursor for cholesterol synthesis but also is a precursor of nonsteroloid isoprenoid compounds, such as geranylgeranylppyrophosphate and farnesylpyrophosphate (4, 59). Geranylgeranylpyporphosphate and farnesylpyrophosphate are implicated in membrane translocation, leading to the activation of a variety of small GTPases such as Ras, Rac, ρ, and Cdc42 (60, 61). ρ, Ras, Rac, and Cdc42 have been shown previously to activate NF-κB (62–64). Inhibitors of ras farnesylation have been shown to suppress IKK activation (65). Alternatively, it is possible that simvastatin suppresses IKK and subsequent NF-κB activation through the inhibition of farnesylation of Ras or other related proteins (4, 66).

We further found that simvastatin blocked the activation of NF-κB without directly interfering with the DNA binding property of NF-κB. Further analysis of the pathway indicated that simvastatin inhibits TNF-α-induced NF-κB activation by inhibiting IKK, which leads to suppression of IκBα phosphorylation and degradation. However, these results differ from those of Holschermann et al. (58), who found that cerivastatin inhibits TNF-α-induced NF-κB activation in endothelial cells through an IKK-independent pathway, suggesting that different statins may mediate their effects through different pathways or that their effects depend on the cell type used.

How simvastatin suppresses IKK activation was also investigated. We found that simvastatin is not a direct inhibitor of IKK. Several kinases such as MEK1 (67), MEK3 (68), protein kinase C (69), glycogen synthase kinase-3β (70), TAK1 (71), PDK1 (72), and Akt (73) have all been reported to function upstream of IKK. Recent studies, however, indicate that TAK1 plays a major role in TNF-α-induced NF-κB activation through its interaction with TAB1 and TAB2. For example, TAK1 can bind and activate IκKβ leading to NF-κB activation (74). TAK1 also has been shown to be recruited by the TNFR1 through TRADD, TRAF2, and RIP (21). Indeed, our study showed for the first time that TAK1-induced NF-κB activation is inhibited by simvastatin. Whether simvastatin modulates other upstream kinases involved in NF-κB activation cannot be excluded based on our studies. RIP mediates the recruitment of TAK1 to TNFR complex and thus plays a very important role in TNF-α-induced IKK activation and subsequent NF-κB activation (51, 53). We found that simvastatin inhibits RIP-induced NF-κB-dependent reporter gene expression.

We further observed that both TNF-α-induced IκBα phosphorylation and degradation were suppressed by simvastatin, leading to inhibition of p65 phosphorylation and translocation into the nucleus. Besides NF-κB activation, the NF-κB-dependent reporter activity induced by TNF-α, TNFR1, TRADD, TRAF2, NIK, and IκKβ was also abrogated by simvastatin. The NF-κB-dependent reporter activity induced by p65, however, was unaffected by simvastatin. These results suggest that the inhibitory effect of simvastatin is downstream of TNF-R1 complexes composed of TRADD/TRAF2/RIP and TAK1/TAB and upstream of p65. Whether simvastatin affects IKK through modulation of NF-κB essential modulator proteins, the regulatory subunit of IKK, is not clear at present. Overall, our data demonstrate that simvastatin is an effective blocker of the NF-κB signaling pathway and thus may have potential in the prevention and treatment of myeloid leukemia and other types of cancers. However, further studies are needed in animals to validate these findings for the therapeutic use of simvastatin.

Acknowledgments

We thank Ann Sutton for carefully reviewing this manuscript. We thank Dr. Madan M. Chaturvedi and other members of the Cytokine Research Laboratory for their useful comments.

Disclosures

The authors have no financial conflict of interest.

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