SPANDIDOS PUBLICATIONS

Pitavastatin inactivates NF-κB and decreases IL-6 production through Rho kinase pathway in MCF-7 cells

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Abstract. The aim of the present study was to provide new mechanistic insight into the effect of pitavastatin at low dose on NF-κB activated by TNF-α in the human breast cancer cell line (MCF-7). We found that treatment of MCF-7 with 1 μM pitavastatin inhibited the proliferation and suppressed the nuclear expression of NF-κB p65 induced by TNF-α with Western blotting. Furthermore, EMSA showed that pitavastatin significantly reduced the DNA binding activity of NF-κB induced by TNF-α. Subsequently, luciferase assay revealed that pitavastatin (1 μM) inhibited the transcriptional activity of the NF-κB promoter, which was clearly related to the HMG-CoA reductase activity because addition of mevalonic acid (MEV) could elevate the NF-κB activity. Moreover, the Rho kinase inhibitor Y27632 abolished the effect of pitavastatin on NF-κB activity. Finally, the addition of TNF-α significantly increased IL-6 protein production, which was suppressed by the addition of pitavastatin. These results suggest that pitavastatin at low dose (1 μM) inhibits NF-κB activation and decreases IL-6 production induced by TNF-α. It is dependent on Rho kinase pathway in human breast cancer cells.

Introduction

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly referred to as statins, are potent inhibitors of cholesterol biosynthesis and widely prescribed for the treatment of hypercholesterolemia. Interestingly, recent studies have been linked with several beneficial effects beyond their effect on cardiovascular desease, including reduction in risk of dementia (1), feratures (2) and cancer (3,4). In addition, the anti-inflammatory action (5), as well as the reduction of pro-inflammatory signaling by statins, such as cytokine and oxygen radical formation, has been reported (6,7). A growing body of evidence suggests that statins may have chemopreventive potential against breast cancer. Laboratory studies demonstrate that statins induce apoptosis and reduce cell invasiveness in various cell lines, including breast carcinoma cells (8). Atorvastatin possessed anticarcinogenic properties in breast cancer cells (9).

A causal relationship between inflammation and cancer has been suspected (10). The molecular and cellular mechanisms linking chronic inflammation to tumorigenesis remain largely unresolved (11). The nuclear factor κB (NF-κB) complex is composed of a family of inducible transcription factors found in almost all cells (12); and this complex is generally recognized as an essential cell mediator acting at the cross-roads of life and death (13). NF-κB activation can become abnormal during organ aging, with development and progression of various chronic inflammatory disorders, and in malignancies such as B and T cell lymphoma and leukemia, and thyroid, head and neck, gastrointestinal, and breast carcinoma (14-16). Moreover, the activation of NF-κB has been shown to play an important role in enhancing the expression of several inflammatory cytokine genes, including TNF-α, IL-6 and IL-8. This phenomenon is observed in various cell types upon stimulation with such agonists as IL-1 and TNF-α (17).

Pitavastatin, is a novel highly potent inhibitor of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (18). The mechanisms of anti-cancer effect by pitavastatin are poorly investigated in breast cancer cells. Therefore, the purpose of the present study was to examine the effect of pitavastatin treatment on human breast carcinoma cells (MCF-7). Here we show that pitavastatin inhibits NF-κB activation and IL-6 protein production induced by TNF-α in human breast carcinoma cells, which is dependent on Rho kinase pathway.

Materials and methods

Reagents. Pitavastatin (trade name: LIVALO®, code name: NK-104) was kindly provided by Kowa Co., Ltd. (Nagoya, Japan) and Nissan Chemical Industries, Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Recombinant TNF-α and Rho kinase inhibitor Y27632 were purchased from Calbiochem

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(Darmstadt, Germany). Mevalonic acid (MEV) was purchased from Sigma (St. Louis, MO, USA).

Cell culture. The human breast carcinoma cell line MCF-7 was originally obtained from the American Type Culture Collection (ATCC) and maintained in RPMI-1640 medium (Sigma) containing 10% fetal bovine serum (FBS). The cells were maintained at 37˚C and 5% CO_2. For experiments, the cells were seeded in 6-well culture plates and grown in complete medium to 90% confluence. Then, the cells were washed with phosphate-buffered saline (PBS) and incubated for the indicated times at 37˚C in 2 ml of medium containing pitavastatin, TNF-α or vehicle, in the presence or absence of MEV, Y27632.

WST-8 assay. The cell proliferation was evaluated using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] colorimetric assay. MCF-7 cells ($1x10^4$ cells/well) were seeded into 96-well plates in 100 μl of culture medium for overnight, and then treated with various concentrations of pitavastatin for 48 h. Next, 10 μl of WST-8 reagent solution (Cell Counting kit, Dojindo Laboratories, Japan) was added and incubated for 2 h. Cell viability was determined according to the manufacturer's instructions.

Extraction of nuclear protein. After treatment of the cells with reagents as described above, nuclear protein was extracted. Briefly, the cells were harvested in 1 ml of ice-cold PBS and centrifuged for 1 min at 5000 rpm at 4˚C. The cell pellet was lysed with 0.4 ml of buffer A containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF), for 15 min on ice. Then, 25 μl of 10% Nonidet P-40 solution was added and the samples were vortexed for 15 sec before centrifuging at 15000 rpm for 5 min at 4˚C. The pellet was washed once with 0.5 ml of buffer A and resuspended in 50 μl of buffer B, which was composed of 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF. The lysed nuclei were left on ice for 30 min and then centrifuged at 15000 rpm for 5 min at 4˚C. The nuclear protein concentration was determined by the DC protein assay (Bio-Rad, Hercules, CA, USA). Nuclear extracts were stored at -80˚C until use.

Western blotting. Aliquots of the nuclear extracts (20 μg of protein each) were separated by 8.5% SDS-PAGE, electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), blocked with 5% non-fat milk in TBS-Tween buffer (20 mM Tris-HCl, pH 7.4, 135 mM NaCl, 0.1% Tween) for 1.5 h at room temperature, and incubated with the appropriate antibody overnight at 4˚C, and then with horseradish peroxidase conjugated secondary antibody for 30 min at room temperature. After extensive washing, immunoreactive proteins were detected with an enhanced chemiluminescence detection system (ECL; Amersham Biosciences Corp., Piscataway, NJ, USA). The following primary antibodies were used: polyclonal antibody for NF-κB p65 from Santa Cruz; and monoclonal antibody for TFIIB from BD Transduction Laboratories.

Electrophoretic mobility shift assay (EMSA). The following reagents were used for the phosphorylation reaction: 3 pmol of consensus oligonucleotide 5'-AGTTGAGGGGACTTTCC CAGGC-3' NF-κB DNA binding sequence (Promega, Madison, WI, USA), 5X T4 polynucleotide kinase buffer, T4 polynucleotide kinase, nuclease-free water and 10 μCi of [γ-^{32}P]-ATP. The reaction mixture was incubated for 10 min at 37˚C. Nuclear extract (8 μg) was incubated in binding buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5) and 1.4 mg/ml poly(dI):poly(dC) containing ^{32}P-labeled NF-κB oligonucleotide with or without the unlabelled oligonucleotide probe at room temperature for 20 min. The sample was loaded on a pre-run 5% polyacrylamide gel. The gel was dried, exposed overnight and analyzed by autoradiography scanning densitometry.

NF-κB luciferase reporter assay. The human 3X κB promoter constructs, NF-κB-dependent luciferase reporter (3X κB-luc), was kindly provided by Dr Shigeki Miyamoto [University of Wisconsin, WI, USA (19)]. MCF-7 cells were seeded onto 6-well plates and cotransfected with 1.0 μg of firefly-luciferase reporter plasmid and 0.1 μg of renilla-luciferase transfection control (phR-CMV; Promega, Southampton, UK) using Lipofectamine™ with Plus Reagents (Invitrogen). After transfection for 24 h, the cells were treated with reagents for indicated times, and then harvested for the luciferase assay. Luciferase activity was measured with the Dual-Luciferase assay system according to the manufacturer's instructions (Promega) and a luminometer (Gene-Light 55, Microtech Nichion, Chiba, Japan). The firefly-luciferase activity of NF-κB was normalized to the renilla-luciferase activity and expressed as fold induction compared with the control value.

Enzyme linked immunosorbent assay (ELISA). Cytokine concentrations in the supernatants of the control and IL-6 treated with reagents for indicated times, were measured using commercial enzyme linked immunosorbent assay (ELISA) kits for IL-6 (Pierce Chemical, Rockford, IL, USA), according to the manufacturer's instructions. Values were expressed as pg/mg protein.

Statistical analysis. Data are presented as means ± SD. Statistical analysis was performed with the Student's t-test.

Results

Growth inhibition of MCF-7 cells by pitavastatin. The clinically used concentrations of pitavastatin in plasma are 0.1-1 μM (18). To elucidate the optimal administration for pitavastatin to MCF-7 cells, the effect of pitavastatin (from 0.1-10 μM) on MCF-7 cell proliferation was examined by the WST-8 assay. Growth inhibitory effects were observed for treatment with pitavastatin at 1-10 μM (Fig. 1) compared with control. Therefore, the inflammatory effects in MCF-7 cells were detected at a low dose of pitavastatin (1 μM).

Pitavastatin inhibits NF-κB p65 expression by TNF-α. There is evidence that the survival of cells and their progression to malignancy are regulated by NF-κB (20). We examined whether pitavastatin inhibits NF-κB by immunoblotting with

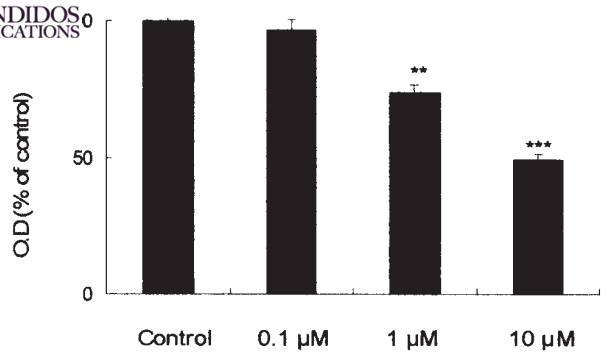

Figure 1. Growth inhibition of pitavastatin in MCF-7 cells. Cells were treated with different concentrations of pitavastatin for 48 h, and the cell growth was determined using a WST-8 colorimetric assay. The results are expressed as percentages of cell growth relative to untreated control cells. The data represent the means ± SD (n=3), **p<0.01, ***p<0.001 vs. control.

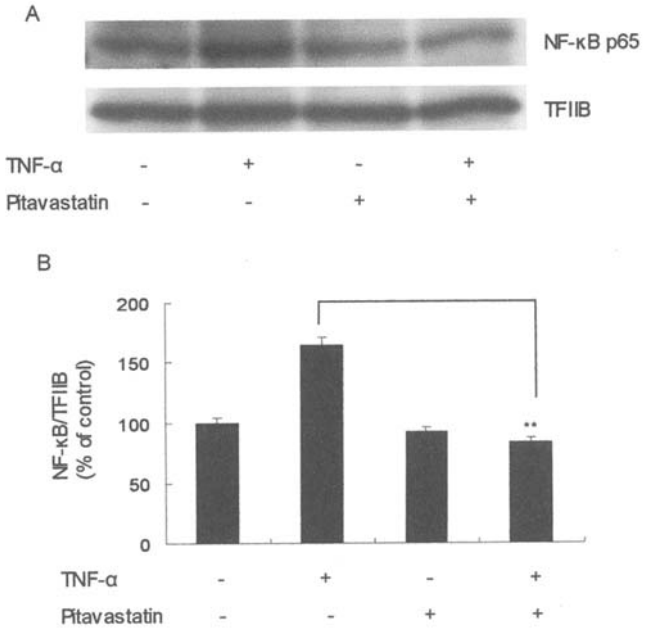

Figure 2. Pitavastatin treatment inhibits NF-κB expression induced by TNF-α in MCF-7 cells. Subconfluent monolayers of MCF-7 cells were treated with pitavastatin (1 μM) alone or pitavastatin (1 μM) and TNF-α (1 ng/ml) for 24 h. After the treatment, the nuclear protein was extracted and NF-κB activation was examined by immunoblotting with an antibody specific for NF-κB or TFIIB. The two panels on top show the protein expression levels of NF-κB and TFIIB (A); bar graphs at the bottom show the expression levels of NF-κB relative to those of TFIIB (B). The data represent means ± SD (n=3), **p<0.01 vs. TNF-α.

an antibody specific for NF-κB p65. TNF-α strongly induced the expression of NF-κB p65 by ~1.6-fold compared with the control in the nuclei of MCF-7 cells by Western blotting. However, the induced NF-κB p65 expression was significantly suppressed by the addition of pitavastatin at 1 μM (p<0.01, Fig. 2).

Pitavastatin reduces DNA binding activity of NF-κB induced by TNF-α. To further investigate the effect of pitavastatin on NF-κB activation in MCF-7 cells, the cells were stimulated with

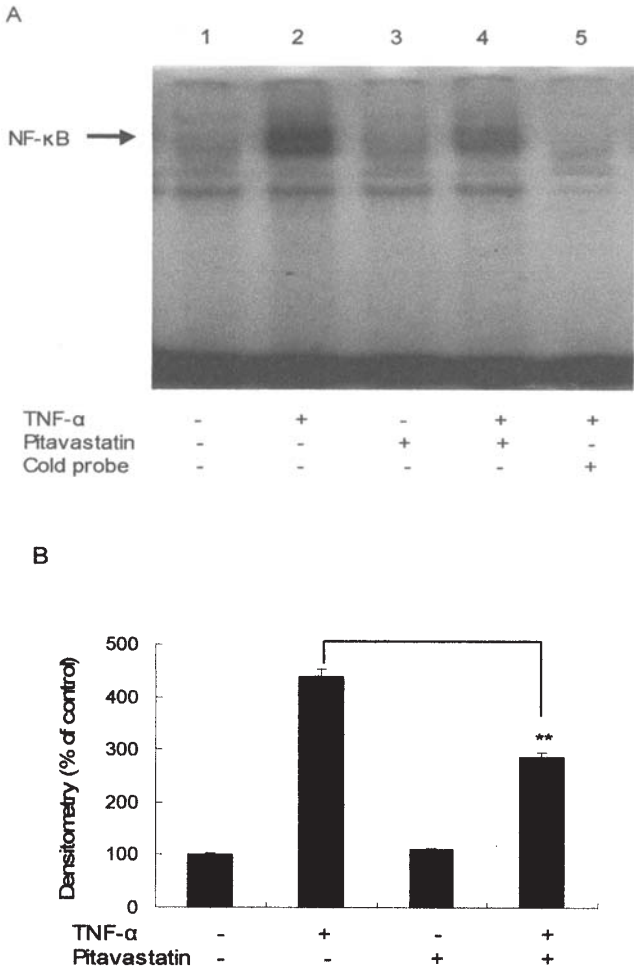

Figure 3. Pitavastatin decreases NF-κB binding to DNA induced by TNF-α in MCF-7 cells. The MCF-7 cells were cultured with pitavastatin (1 μM), TNF-α (1 ng/ml) or pitavastatin plus TNF-α. Nuclear extracts were extracted, and then subjected to electrophoretic mobility shift assay (EMSA). EMSA flourograms (A) and densitometric patterns (B). The resulting complexes were separated on 5% non-denaturing polyacrylamide gel. To test for the specificity of the NF-κB-DNA binding, a competition experiment with 25-fold excess of unlabeled oligonucleotide was carried out. The data represent means ± SD (n=3), **p<0.01 vs. TNF-α.

pitavastatin alone or with pitavastatin and TNF-α for 24 h. Nuclear extracts were prepared and analyzed by EMSA. As shown in the EMSA fluorograms (Fig. 3A) and the densitometric patterns (Fig. 3B), NF-κB activation in the MCF 7 cells was increased by ~4.5-fold after TNF-α stimulation compared with the control and the addition of pitavastatin significantly inhibited NF-κB binding to DNA induced by TNF-α (p<0.01).

Pitavastatin decreases NF-κB promoter transcriptional activity induced by TNF-α. To further investigate the effect of pitavastatin on NF-κB promoter activity, we performed the dual-luciferase assay with a reporter vector containing the NF-κB promoter in MCF-7 cells. As shown in Fig. 4A, upon stimulation with TNF-α, the luciferase reporter activity was increased by ~2.5-fold compared with control. Pitavastatin at 1 μM significantly inhibited the NF-κB promoter transcriptional activity induced by TNF-α.

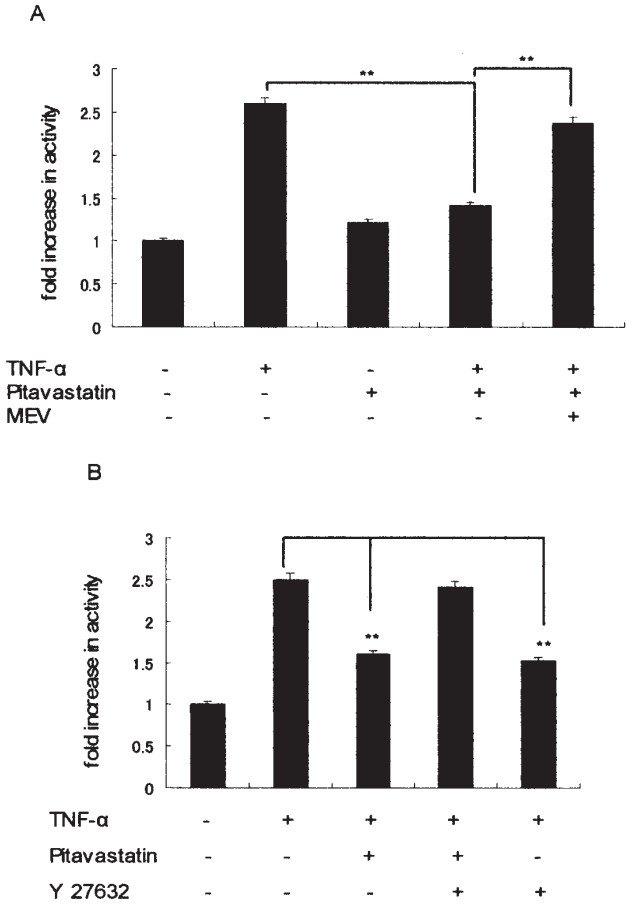

Figure 4. Pitavastatin reduces the transcriptional activity of the NF-κB promoter induced by TNF-α dependent of Rho kinase pathway in MCF-7 Cells. Cells plated in 6-well plates were transfected with 3xκB-luc plasmid. One day after the transfection, the cells were treated with or without pitavastatin (1 μM), or TNF-α (1 ng/ml), or MEV (1 mM) for 24 h (A); or with pitavastatin (1 μM) alone or in combination with TNF-α, or Y27632 (10 μM) for 24 h (B), and then luciferase activity in the cell lysates was measured. The experiments were performed in triplicate. The data represent means ± SD (n=3), $^{**}p<0.01$.

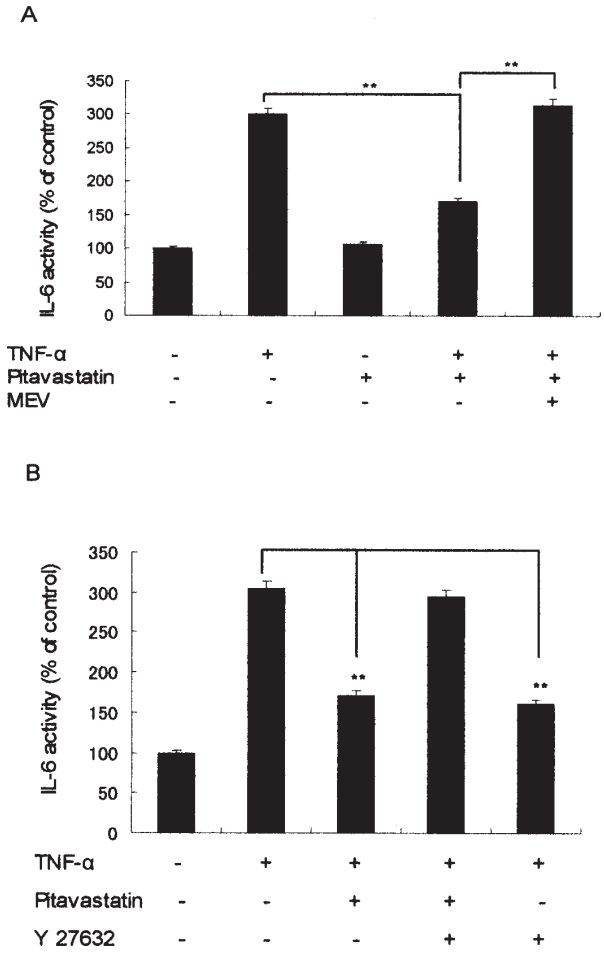

Figure 5. Pitavastatin decreases IL-6 production induced by TNF-α in MCF-7 cells. Confluent MCF-7 cells were incubated for 24 h with or without pitavastatin (1 μM), or TNF-α (1 ng/ml), or MEV (1 mM) (A); or with pitavastatin (1 μM) alone or in combination with TNF-α, or Y27632 (10 μM) for 24 h (B). Concentrations of IL-6 in the supernatants were measured by ELISA. Bars represent means ± SD (n=3), $^{**}p<0.01$.

Inactivation of NF-κB by pitavastatin is dependent on the Rho kinase pathway. We further examined the potential mechanism underlying the pitavastatin inhibitory effect on NF-κB activity by luciferase assay. Generated by HMG-CoA reductase, L-mevalonate serves as a key intermediate of cholesterol synthesis from acetyl-CoA. To determine whether the pitavastatin inhibition of NF-κB activity occurred through blocking the HMG-CoA reductase activity or reduction in L-mevalonate synthesis, we added exogenous MEV (1 mM) into the cultures of MCF-7 cells with TNF-α in the presence or absence of pitavastatin (1 μM). The pitavastatin-suppressed NF-κB transcriptional activity was clearly related to the HMG-CoA reductase activity because addition of MEV could elevate the NF-κB activity (Fig. 4A).

In addition, the Rho family of small GTP-binding proteins consists of three subfamilies, Rho, Rac and Cdc42, which play important roles in signal transduction and inflammation (21,22). Treatment with statin reduces Rho GTPase activation, thereby diminishing monocytes stable in modulation to vascular endothelium (23). The Rho kinase inhibitor Y27632 specifically inactivates p160 Rho-associated protein kinase (p160ROCK), a key subunit of this kinase, known to regulate NF-κB activity (24). We tested whether Y27632 could mimic the inhibition of pitavastatin on NF-κB activity by TNF-α in MCF-7 cells. The Rho kinase inhibitor Y27632 abolished the effect of pitavastatin on NF-κB activity (Fig. 4B). In addition, Y27632 decreased the NF-κB activity by TNF-α (Fig.4B). These results suggest that the Rho signaling pathway may participate in pitavastatin inhibition of NF-κB activity.

Pitavastatin decreases IL-6 protein production. The activation of NF-κB has been shown to play an important role in enhancing the expression of several cytokine genes, including IL-6 and IL-8 (17). It has been reported that serum levels of IL-6 are highly elevated in breast cancer patients and correlate with tumor progression (25). To evaluate the effect of pitavastatin on the production of the inflammatory marker, we measured IL-6 protein production in the supernatant of MCF-7 cells by ELISA. The addition of TNF-α significantly increased IL-6 protein production in the MCF-7 cells by ~3-fold compared with control (Fig. 5A); however, the increase was suppressed by 50% upon the addition of pitavastatin. As expected, MEV also elevated low dose pitavastatin-reduced production of IL-6 (Fig. 5A). The Rho

ibitor, Y27632 diminished the effect of pitavastatin roduction (Fig. 5B).

Discussion

Breast cancer is the most common cancer of women worldwide. Its frequency increases with age (26). That there is a connection between inflammation and cancer has been suspected for a long time (10,11). Inflammatory cells have been largely ignored as a promoter of tumor development. More importantly, proliferation in the setting of chronic inflammation predisposes humans to carcinoma of the large bowel, liver, breast, urinary bladder, gastric mucosa, prostate, ovary and skin (27).

There is growing evidence that statins exert anti-inflammatory and antioxidative vascular actions that are independent of their serum lipid lowering effects (28). The anti-inflammatory action (5), as well as the reduction of pro-inflammatory signaling, such as cytokines and oxygen radical formation (6,7), by statin treatment has been reported.

There is a long-standing debate concerning the association between use of statins and cancer. Several recent laboratory studies indicated that statins may have chemopreventive potential against cancer at various sites including breast cancer (29). Lovastatin (1-10 μM) inhibited the proliferation of MCF-7 cells by inducing apoptosis and G1/S cell cycle arrest (30). In the present study, pitavastatin at a low dose (1 μM) induced an anti-inflammatory effect through inactivation of NF-κB in MCF-7 cells with anti-proliferation.

Previously, we found that NF-κB expression is necessary for the maintenance of the malignant phenotype and provides a therapeutic approach to cancer (31). Activated NF-κB has been detected in different tumor cell lines and primary tumor samples (32). Moreover, the activation of NF-κB has been shown to play an important role in enhancing the expression of several cytokine genes, including IL-6 and IL-8. This phenomenon has been observed in various cell types upon stimulation with such agonists as IL-1 and TNF-α (17). It has been reported also that statin treatment inhibits NF-κB activation in differential cell lines. For example, treatment with atorvastatin inhibited the DNA binding activity of NF-κB in arterial smooth muscle cells (33). Gel shift assay revealed that cerivastatin (25 ng/ml) displayed a potent anti-invasive effect on human breast cancer cells (MDA-MB-231 cells), which could be related to NF-κB inhibition (34). The anti-inflammatory action of simvastatin (3 μM) on human C-reactive protein (huCRP) expression in hepatocytes is based on the up-regulation of the cytosolic inhibitor of NF-κB, IκBα, resulting in reduced NF-κB activity (35). Previously, it was reported that pitavastatin (5-15 μM) decreased NF-κB p65 expression by Western blotting and inhibited NF-κB activity by EMSA in macrophages (36). Our data showed that pitavastatin at a low dose (1 μM) could markedly inhibit the TNF-α-induced NF-κB activation in MCF-7 cells. Furthermore, pitavastatin significantly decreased the DNA binding activity of NF-κB. Subsequently, we analyzed the effect of pitavastatin on NF-κB-dependent gene expression by reporter gene analysis using an NF-κB-specific minimal promoter construct, and found that the increase in NF-κB-regulated gene expression was largely blocked upon treatment of the cells with pitavastatin, closely approximating the results of Western blotting. To assess whether the down-regulation of NF-κB protein expression was mediated by the degradation of IκBα expression by pitavastatin, IκBα expression was determined in the cytosolic protein of MCF-7 by Western blotting. We observed no significant effect on IκBα expression levels compared with control (data not shown). These results suggest that the inactivation of NF-κB by a low dose of pitavastatin is not dependent on IκBα degradation.

The Rho families of GTPases are involved in the regulation of multiple cellular functions including cell migration, proliferation and inflammation (21,22). Some studies have demonstrated that statins may regulate the cytokine-induced IL-6 level through different signal pathways that involved both Y27632-sensitive and insensitive molecular signaling, in particular the NF-κB pathway (24). Pitavastatin (10 μM) has been suggested to have an anti-inflammatory role through the RhoA-dependent pathway in monocytic THP-1 cells (37). Our results demonstrate that the effect of pitavastatin at 1 μM appears to be specific to the inhibition of HMG-CoA reductase, as the addition of MEV abolishes the inhibitory effect of pitavastatin. Furthermore, the pitavastatin-reduced NF-κB activity is dependent on the Rho kinase pathway. To establish the exact role of the small GTP-binding proteins in the regulation of NF-κB activity by pitavastatin in the MCF-7 cells, it is essential to examine the effect of the dominant-negative mutants of the small GTP-binding proteins.

IL-6 is a pleiotropic cytokine with obvious tumor-promoting and -inhibitory effects (38). It has been reported that serum levels of IL-6 are highly elevated in breast cancer patients and correlate with tumor progression (25). To evaluate the effect of pitavastatin on the production of the inflammatory marker, we measured the amount of IL-6 protein produced in the supernatant of MCF-7 cells. The TNF-α-induced IL-6 production was suppressed by the addition of pitavastatin, which was abolished by MEV and mediated Rho kinase pathway. Our findings demonstrate that pitavastatin not only inhibits the activation of NF-κB, but also decreases the expression level of the pro-inflammatory cytokine IL-6, which is dependent on Rho kinase pathway, which may influence the evolution of the human breast cancer inflammatory process.

It has been reported that pitavastatin is a powerful statin and has favorable pharmacokinetic properties. Specifically, it is selectively localized to the liver, and <5% of a given dose reaches the systemic circulation (39). Therefore, our study provides important *in vitro* evidence that pitavastatin at low dose (1 μM) exerts an anti-cancer effect by inhibiting NF-κB activation independent of cholesterol reduction, very close to the expected plasma levels of it in patients, and may also potentially play a beneficial role in cancer therapy.

In summary, pitavastatin at 1 μM decreased the production of the pro-inflammatory cytokine, IL-6, through the inhibition of NF-κB activation in MCF-7 cells. The effects of pitavastatin may be dependent on Rho kinase pathway, independent of its serum lipid lowering effect.

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