RESEARCH ARTICLE

Naringin inhibits matrix metalloproteinase-9 expression and AKT phosphorylation in tumor necrosis factor-α-induced vascular smooth muscle cells

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Citrus fruits are high in naringin, which has a beneficial effect on cardiovascular diseases. However, the matrix metalloproteinase-9 (MMP-9) regulation involved in cell migration and invasion remains to be identified. Naringin inhibited tumor necrosis factor-α (TNF-α)-induced expression of MMP-9, under 10–25 μM concentration conditions in vascular smooth muscle cells (VSMC). The TNF-α-induced invasion and migration of VSMC were inhibited by naringin. Furthermore, naringin suppressed TNF-α-mediated release of interleukin-6 and -8 (IL-6 and IL-8). However, naringin (10–25 μM) treatment of VSMC in the presence of TNF-α did not affect cell growth and apoptosis. In additional experiments, naringin reduced the transcriptional activity of activator protein-1 and nuclear factor kappaB (NF-κB), which are two important nuclear transcription factors that are involved in MMP-9 expression. Also, naringin treatment blocked PI3K/AKT/mTOR/p70S6K pathway in TNF-α-induced VSMC. Treatment of aglycone naringenin (10–25 μM) had same effect on the levels of MMP-9 expression, invasion, migration, and AKT phosphorylation in TNF-α-induced VSMC, compared with naringin treatment. These results suggest that naringin represses PI3K/AKT/mTOR/p70S6K pathway, invasion and migration, and subsequently suppresses MMP-9 expression through the transcription factors NF-κB and activator protein-1 in TNF-α-induced VSMC. These novel findings provide a theoretical basis for the preventive use of naringin for atherosclerosis disease.

Keywords:
MMP-9 / Naringin / PI3K/AKT/mTOR/p70S6K pathway / TNF-α / Vascular smooth muscle cell

1 Introduction

The development of atherosclerotic lesions is characterized by excessive vascular remodeling with accumulation of cells and lipids within the intimal layer of the pathological artery [1, 2]. Increased proteolytic activity in the vessel wall mediates the degradation of the extracellular matrix surrounding smooth muscle cells in response to injury [3], a necessary step to permit medial smooth muscle cells to migrate into the intimal space. The gelatinases MMP-2 (72 kDa) and MMP-9 (92 kDa) are matrix metalloproteinases (MMPs) that have been implicated as mediators of lesion development during atherosclerosis, the structural reorganization of the blood vessel [3]. MMP-2 is constitutively expressed by several cell types, including smooth muscle cells, and its expression is not induced by cytokines or growth factors [4, 5]. Expression of MMP-9 is precisely controlled by various stimuli, including tumor necrosis factor-α (TNF-α), but not platelet-derived

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Abbreviations: AP-1, activator protein-1; **MAPK,** mitogen-activated protein kinase; **MMP,** matrix metalloproteinase; **TNF,** tumor necrosis factor; **VSMC,** vascular smooth muscle cell

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growth factor or thrombin [4–6]. Many studies indicate that MMP-9 plays a pluripotent role in pathological events such as atherosclerosis. For example, MMP-9 is present in low amounts in normal arteries, and is upregulated within 6 h after injury in rat carotid arteries and continues to be expressed for up to 6 days [7]. Additional animal experiment (MMP-$9^{-/-}$) data indicate that MMP-9 is critical for the development of arterial lesions by regulating both vascular smooth muscle cells (VSMC) migration and proliferation [8].

Vascular lesions form during several pathological processes, which involve the accumulation of inflammatory cells and the release of cytokines [9]. TNF-α is a cytokine secreted by VSMC in the neointima after balloon injury, as well as by macrophages in atherosclerotic lesions [10–12]. Previous studies have indicated that TNF-α is a potent activator of MMP-9 in various cell lines [13–16]. Analysis of MMP-9 promoter activity has been shown that it contains essential proximal activator protein-1 (AP-1) and NF-κB binding sites [13–16]. Activation of the mitogen-activated protein kinases (MAPK) and PI3K/AKT signaling pathways [6, 16–18] have been shown to mediate MMP-9 expression.

Naringin is a major constituent of the flavonoid in citrus fruits. Epidemiological studies suggest that naringin exerts beneficial effects as an anti-cancer, anti-oxidative, and anti-atherogenic agent in animal studies [19–23]. Although many studies have analyzed the effects of naringin on growth inhibition in various cell lines [19–21], the MMP-9 regulation involved in the inhibition of cell migration and invasion remains to be identified.

The purpose of the present study is to examine the roles of MMP-9 expression and the PI3K/AKT signaling pathway in the regulation of naringin-induced inhibition of VSMC invasion and migration.

2 Materials and methods

2.1 Materials

Naringin and naringenin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Naringin was dissolved in DMSO and stored as a 1 M stock solution. It was used after dilution of the stock solution with DMSO. TNF-α was obtained from R&D systems. Polyclonal antibodies to AKT, phospho-AKT, ERK, phospho-ERK, p38 MAP kinase, phospho-p38 MAP kinase, JNK, phospho-JNK, phospho-mTOR, and phospho-p70S6K were obtained from Cell signaling (Beverley, MA, USA). Wortmanin, PD98059, SP600125, and SB203580 were obtained from Calbiochem (San Diego, CA, USA). Polyclonal MMP-9 antibody was obtained from Chemicon.

2.2 Cell cultures

VSMC were isolated from Sprague–Dawley rats. These explants were grown in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 50 μg/mL gentamycin, and 50 μL/mL amphotericin-B at 37°C in a humidified 5% CO_2 atmosphere.

2.3 Cell viability assay

VSMC, grown to near confluence in 24-well tissue culture plates, were made quiescent with serum-free medium and treated with naringin. Cell viability was determined using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, as described previously [24].

2.4 [^{3}H]thymidine incorporation

VSMC, grown to near confluence in 24-well tissue culture plates, were made quiescent and treated with naringin, as indicated. The cells were incubated for an additional 24 h and labeled with [methyl-^{3}H]thymidine (New England Nuclear, Boston, MA, USA) at 1 μCi/mL during the last 12 h of this time period. After labeling, the cells were washed with phosphate-buffered saline and fixed in cold 10% trichloroacetic acid and then washed with 95% ethanol. The incorporated [^{3}H]thymidine was extracted with 0.2 M NaOH and the radiochemical activity determined as described previously [16, 24]. Values are expressed as the mean of six wells from three separate experiments.

2.5 Apoptosis detection by ELISA

This method is based on a quantification of the enrichment of mono- and oligo-nucleosomes in the cytoplasm by Cell Death Detection ELISA kit (Roche, Mannheim, Germany) [24].

2.6 Invasion assay

VSMC were resuspended with TNF-α (100 ng/mL) alone or together with naringin in 100 μL of medium and placed in the upper part of the transwell plate. The cells were then incubated for 24 h. Cells had to pass an 8 μM pore size polycarbonate membrane with a thin layer of ECMatrix-likewise material. The ability of VSMC to invade the ECMMatrix-likewise material was determined with a commercial cell invasion assay kit (Chemicon International), as described previously [25].

2.7 Wound migration assay

VSMC were plated on a six-well dishes and grown to 90% confluence in 2 mL of growth medium. The cells were damaged using a 2-mm-wide tip. The cells were treated with TNF-α (100 ng/mL) alone or together with naringin. The cells were allowed to migrate, and photographs were taken through an inverted microscope (× 40 magnification).

2.8 Immunoblotting assays

Growth-arrested VSMC were treated with TNF-α in the presence or absence of naringin for the specified time periods at 37°C. Cell lysates were prepared, and immunoblotting was performed as described previously [16, 24].

2.9 Transient transfection

Each plasmid was transfected into VSMC using a Superfect reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions [16]. Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Firefly luciferase activity was standardized to β-galactosidase activity.

2.10 Zymography

The conditioned medium was electrophoresed in a polyacrylamide gel containing 1 mg/mL gelatin. The gel was then washed at room temperature for 2 h with 2.5% Triton X-100 and then at 37°C overnight in a buffer containing 10 mM $CaCl_2$, 150 mM NaCl, and 50 mM Tris–HCl, pH 7.5. The gel was stained with 0.2% Coomassie blue and photographed on a light box. Proteolysis was detected as a white zone in a dark blue field.

2.11 Creation of MMP-9 promoter reporter construct

A 0.7 kb segment at the 5′-flanking region of the human MMP-9 gene was amplified by PCR using specific primers from the human MMP-9 gene (Accession No. D10051): 5′-ACATTTGCCCGAGCTCCTGAAG (forward/SacI) and 5′-AGGGGCTGCCAGAAGCTTATGGT (reverse/Hind III). The pGL2-Basic vector containing a polyadenylation signal upstream from the luciferase gene was used to construct the expression vectors by subcloning PCR-amplified DNA of the MMP-9 promoter into the SacI/HindIII site of the pGL2-Basic vector [16]. The size of the PCR products was confirmed by electrophoresis and by DNA sequencing.

2.12 Nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts were prepared essentially as described elsewhere [16]. Cultured cells were collected by centrifugation, washed, and suspended in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF. After 15 min on ice, the cells were vortexed in the presence of 0.5% Nonidet NP-40. The nuclear pellet was then collected by centrifugation and extracted in a buffer containing 20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1mM PMSF for 15 min at 4°C.

The nuclear extract (10–20 μg) was preincubated at 4°C for 30 min with a 100-fold excess of an unlabeled oligonucleotide spanning the −79 MMP-9 cis element of interest. The sequences were as follows: AP-1, CTGACCCCTGAGTCAGCACTT; NF-κB, CAGTGGAATTCCCCAGCC. The reaction mixture was then incubated at 4°C for 20 min in a buffer (25 mM Hepes buffer, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl, and 2.5% glycerol) with 2 μg of poly dI/dC and 5 fmol (2×10^4 cpm) of a Klenow end-labeled (^{32}P-ATP) 30-mer oligonucleotide, which spans the DNA-binding site in the MMP-9 promoter. The reaction mixture was electrophoresed at 4°C in a 6% polyacrylamide gel using a TBE (89 mM Tris, 89 mM boric acid, and 1 mM EDTA) running buffer. The gel was rinsed with water, dried, and exposed to an X-ray film overnight.

2.13 Measurement of IL-6 and IL-8

IL-6 and IL-8 released into the culture medium were determined by enzyme-linked immunosorbent assay kit (ELISA, Endogen, Wobrun, MA, USA).

2.14 Statistical analysis

When appropriate, data were expressed as mean ± SE. Data were analyzed by factorial ANOVA and Fisher's least significant difference test where appropriate. Statistical significance was set at $p < 0.05$.

3 Results

3.1 Naringin reduces VSMC proliferation

VSMC were grown in TNF-α-containing medium (100 ng/mL) in the absence or presence of concentrations of naringin (5–50 μM) for 24 h. TNF-α stimulated the proliferation of VSMC, as measured by cell viability and DNA synthesis, which did not affect the viability or DNA synthesis for 24 h (Fig. 1A and B). Inhibition of cell growth was observed at 75 μM naringin concentration (Fig. 1A). The vehicle (DMSO) had no effect on basal cell viability (Fig. 1A and B). Upon TNF-α stimulation, the vehicle did not affect cell viability or thymidine incorporation (Fig. 1A and B). Next, we measured naringin-induced apoptosis in VSMC in response to TNF-α. As shown in Fig. 1C, using a quantitative assay, more than 75 μM naringin significantly increased the cytoplasmic DNA–histone complex in 5637 cells.

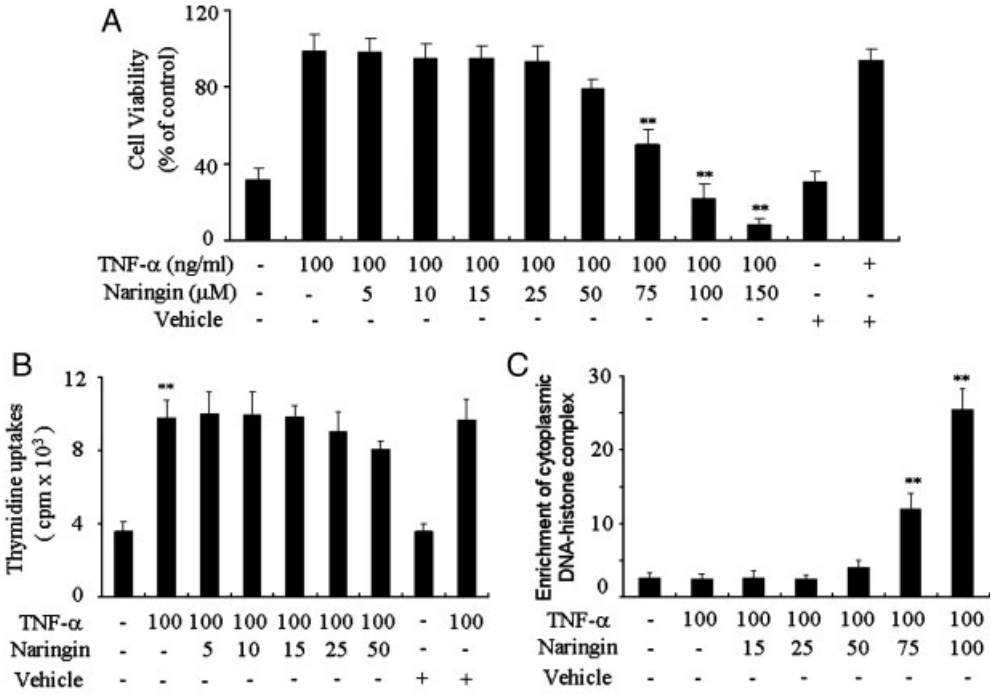

Figure 1. Effects of naringin on cell proliferation and apoptosis in VSMC. After a 1-day period of starvation, the cells were pretreated with naringin at the indicated concentrations (μM) for 30 min before stimulation with or without TNF-α (100 ng/mL) for 24 h, and then harvested. (A) Cell viability was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. $^{**}p<0.05$ compared with TNF-α treatment. (B) Measurement of DNA replication by thymidine uptake as a marker for cell proliferation. $^{**}p<0.05$ compared with no TNF-α treatment. (C) Detection of apoptosis in cells treated with naringin. Quiescent VSMC were stimulated with TNF-α (100 ng/mL) in the presence or absence of the indicated concentration of naringin and cultured for 24 h. The cytoplasmic DNA–histone complex was measured by ELISA. Results are presented as mean ± SE from three triplicate experiments. $^{**}p<0.01$ compared with no naringin treatment in TNF-α-stimulated cells.

3.2 Naringin inhibits TNF-α-induced MMP-9 expression

In the past several years, a number of studies have demonstrated that MMP-9 is important for VSMC proliferation and migration into the intima [2, 3]. Recent studies have reported that TNF-α stimulates the induction of MMP-9 in VSMC [4–6, 16]. Gelatin zymography was used to determine the effects of naringin on gelatinase secretion from VSMC induced by TNF-α. Media from control smooth muscle cells did not demonstrate any proteolytic activity at 92 kDa, corresponding to MMP-9. Treatment with 100 ng/mL of TNF-α induced proteolytic MMP-9 activity, as evidenced by the presence of a band (Fig. 2A). Naringin treatment significantly reduced TNF-α-induced MMP-9 secretion in a dose-dependent manner (Fig. 2A). On the contrary, MMP-2 was constitutively secreted and was not affected by TNF-α or naringin treatment (Fig. 2A). Similar results were found using immunoblot (Fig. 2A). In addition, treatment with naringenin resulted in same effect of MMP-9 expression, compared with the naringin-treated cells (Fig. 2A).

3.3 Naringin attenuates TNF-α-induced invasion and migration of VSMC

Previous studies have suggested that expression of MMP-9 plays a pivotal role in the invasion and migration of VSMC, which are strongly linked to atherosclerosis [4–7]. We next examined whether the inhibition of MMP-9 by naringin is involved in the inhibition of invasion and migration. As shown in Fig. 2B and C, the invasion and migration of VSMC was increased by treatment with TNF-α compared with untreated control cells. Treatment with naringin (at concentrations of 25 μM) strongly inhibited TNF-α-induced invasion and migration (Fig. 2A and B). Moreover, under similar experimental conditions, naringenin treatment showed same levels in migration and invasion, compared with naringin-treated cells (Fig. 2B and C).

3.4 Naringin inhibits the MMP-9 promoter by decreasing the NF-κB and AP-1 binding activities

To better understand the mechanism for decreased MMP-9 expression, we examined MMP-9 promoter activity before

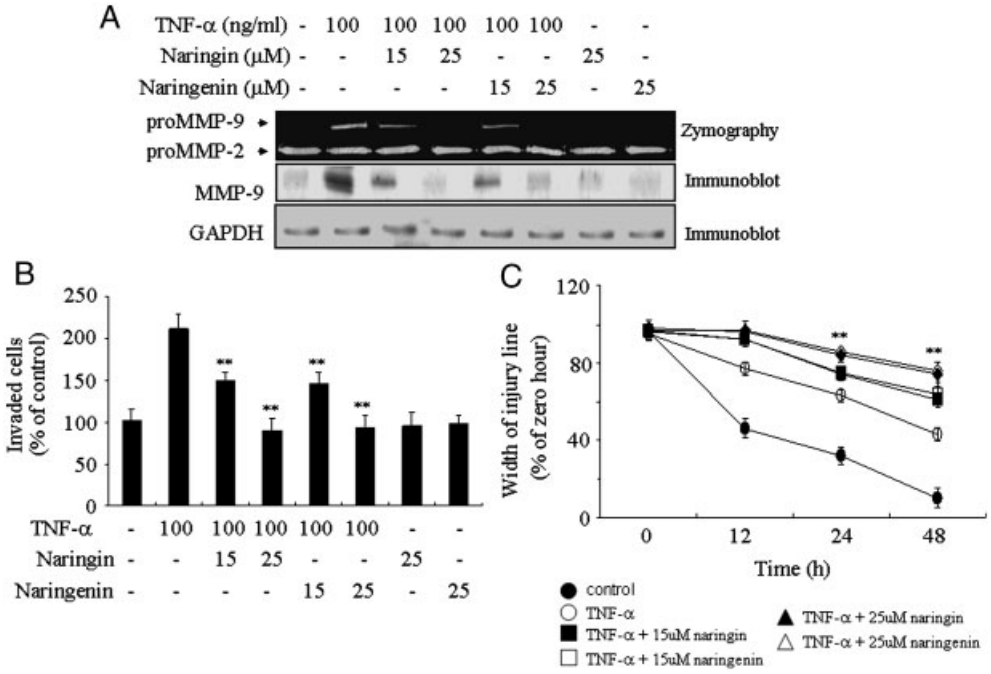

Figure 2. Effects of naringin on TNF-α-induced MMP-9 expression, invasion and migration of VSMC. (A) After a 1-day period of starvation, the cells were pretreated with naringin or naringenin at the indicated concentrations (μM) for 30 min before stimulation with or without TNF-α (100 ng/mL) for 24 h, and then the culture supernatants were analyzed zymographically for MMP activities. Similarly, an immunoblot analysis was performed with antibodies specific for MMP-9. (B) VSMC seeded in serum-free medium were exposed to 10, 15, and 25 μM naringin or naringenin for 30 min before the addition of TNF-α (100 ng/mL) for 48 h. Results are expressed as the number of invaded cells relative to untreated control, as determined from three independent experiments. $^{**}p<0.05$ compared with TNF-α treatment. (C) The confluent VSMC in serum-free medium were exposed to 10, 15, and 25 μM naringin or naringenin for 30 min before the addition of TNF-α (100 ng/mL). The widths of injury lines made in cells were then measured at 0, 12, 24, and 48 h. Results are expressed as the widths of injury lines relative to untreated controls at 0 h, as determined from three independent experiments. $^{**}p<0.05$ compared with TNF-α treatment.

and after TNF-α stimulation in the absence or presence of naringin. In a previous study, we reported that the minimal response elements for TNF-α stimulation, NF-κB and AP-1, are located in the region −710 bp upstream of the transcription start site in VSMC [16, 26]. Therefore, we used a plasmid containing a luciferase reporter gene driven by a 710 bp segment from the 5′-promoter region of a human MMP-9 gene to examine TNF-α-mediated MMP-9 promoter activation in the absence or presence of naringin. VSMC were transiently transfected with the pGL2-MMP-9WT plasmid (see ''Materials and methods'' for details), and subsequently treated with TNF-α for 24 h. TNF-α strongly increased the reporter activity, which can be attributed to the MMP-9 promoter sequence in VSMC (Fig. 3B). In addition, this TNF-α-stimulated MMP-9 promoter activity was reduced by over 90% following the treatment of VSMC with naringin, suggesting that the repressive effect of the naringin is due, at least in part, to reduced transcription of the MMP-9 gene. As mentioned above, our previous studies showed that TNF-α induces MMP-9 expression through the NF-κB and AP-1 cis-elements in VSMC [16, 26]. To determine whether the repressive effect of naringin on MMP-9 expression was mediated through these two types of motifs,

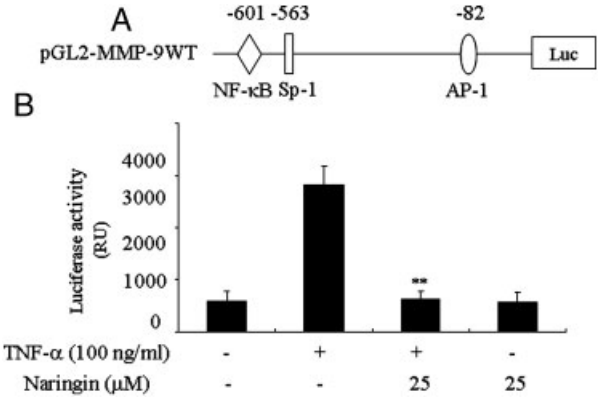

Figure 3. Inhibitory effect of naringin on TNF-α-induced MMP-9 promoter activity in VSMC. (A) Schematic map of the MMP-9 promoter showing the cis-regulatory elements. (B) VSMC were transiently transfected with pGL2-MMP-9WT, which contains 710 bp of the 5′-promoter region of the MMP-9 gene and then cultured with TNF-α (100 ng/mL) in the presence or absence of the indicated concentrations of naringin. Luciferase activity was determined from cell lysates as described in Section 2. $^{**}p<0.05$ compared with TNF-α treatment.

EMSA was performed on the nuclear extracts of VSMC following treatment with TNF-α in the absence or presence of naringin. In EMSA, the nuclear extracts were incubated with a radiolabeled double-stranded oligonucleotide probe containing the consensus sequence for NF-κB and the AP-1 binding site, respectively, and electrophoresed in a 5% nondenaturing polyacrylamide gel. An oligonucleotide derived from the MMP-9 promoter sequence spanning this motif was bound specifically to the nuclear factor derived from the treatment of VSMC with TNF-α (Fig. 4). Nuclear extracts from VSMC treated with TNF-α showed increased binding to NF-κB and AP-1 motifs (Fig. 4). Thus, naringin effectively suppressed the increased NF-κB and AP-1 binding activities (Fig. 4). These data suggest that naringin blocks MMP-9 expression, at least in part, by decreasing the binding of transcription factors NF-κB and AP-1 to DNA.

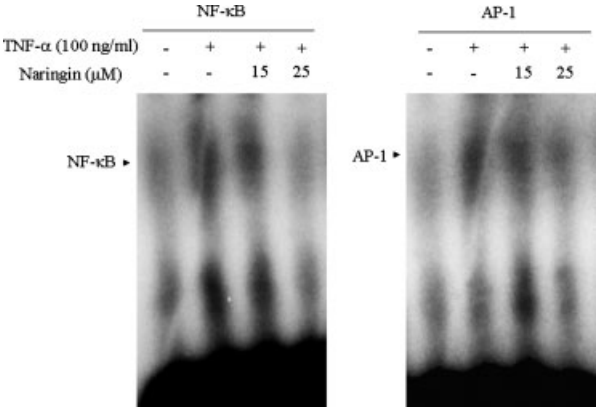

Figure 4. Effects of naringin on DNA binding activities of NF-κB and AP-1 motifs derived from the MMP-9 promoter in TNF-α-induced VSMC. Cells pretreated with the indicated concentrations of naringin for 40 min in serum-free medium were incubated with TNF-α (100 ng/mL) for 24h. After incubation, nuclear extracts from the cells were analyzed by EMSA for activated NF-κB and AP-1 using radiolabeled oligonucleotide probes.

3.5 Naringin prevents TNF-α-induced PI3K/AKT/mTOR/p70S6K pathway

Recent studies have shown that MAP kinase and AKT signaling pathways are strongly involved in TNF-α-induced MMP-9 expression and migration in VSMC [6, 16–18]. To determine whether naringin affects MAP kinase activation in VSMC, time course experiments were performed, measuring ERK1/2, JNK, and p38 MAP kinase activation by TNF-α stimulation in the absence or presence of naringin. Naringin had no effect on TNF-α-induced ERK1/2, JNK, and p38 MAP kinase activation (Fig. 5A–C). We next examined the effect of naringin on the TNF-α-induced AKT phosphorylation as the downstream effector of PI3-kinase. TNF-α-induced AKT phosphorylation was inhibited by naringin in a concentration-dependent manner (15–25 μM) compared to the control (Fig. 5D). Phosphorylation of AKT showed same level on naringenin-treated cells compared with naringin-treated cells (Fig. 5D). TNF-α-induced phosphorylation of MAP kinases was inhibited by PD98059 (MEK inhibitor), SB203580 (p38 MAP kinase inhibitor), and SP600125 (JNK inhibitor) (Fig. 5A–C). Wortmannin (PI3-kinase inhibitor) treatment also prevented TNF-α-induced AKT phosphorylation (Fig. 5D). In addition, the phosphor-

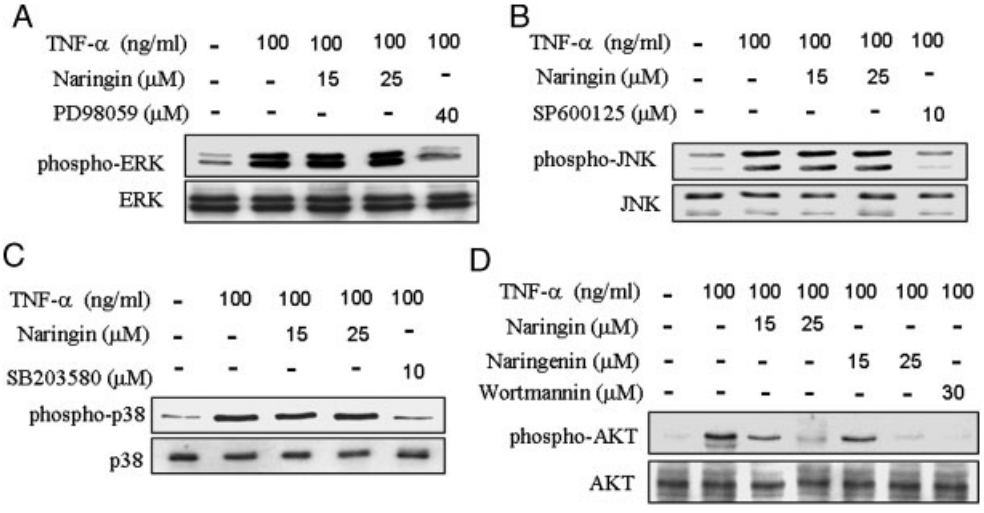

Figure 5. Effects of naringin on ERK1/2, p38, JNK, and AKT phosphorylation. Quiescent VSMC were stimulated with TNF-α (100 ng/mL) in the presence or absence of the indicated concentration of naringin or naringenin at 10 min. The phosphorylations or levels of (A) ERK1/2, (B) JNK, (C) p38, and (D) AKT proteins were detected by immunoblot analysis, using antibodies phosphospecific or specific for either ERK1/2, JNK, p38, or AKT. VSMC were also pretreated for 40 min with PD98059 (40 μM), SP600125 (10 μM), SB203580 (10 μM), and wortmannin (30 μM) before cells were stimulated with TNF-α (100 ng/mL). The phosphorylations or levels of (A) ERK1/2, (B) JNK, (C) p38, and (D) AKT proteins were detected by immunoblot analysis using antibodies phosphospecific or specific for ERK1/2, JNK, p38, or AKT.

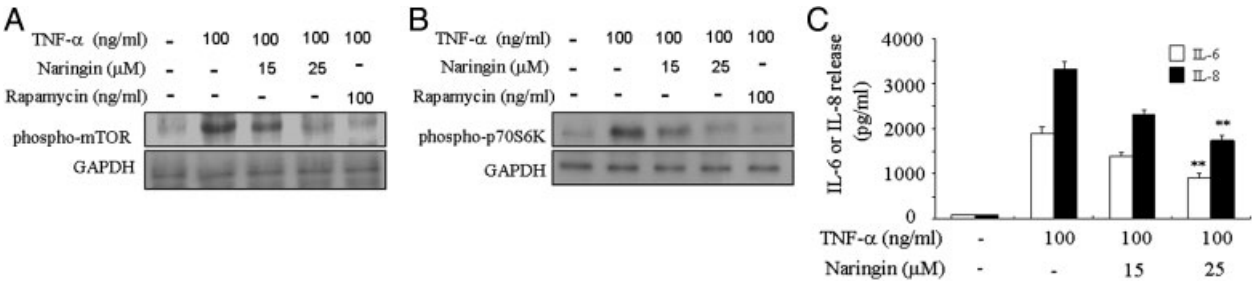

Figure 6. Effects of naringin on mTOR/p70S6K pathway and production of IL-6 and IL-8. Quiescent VSMC were stimulated with TNF-α (100 ng/mL) in the presence or absence of the indicated concentration of naringin at 10 min. VSMC were also pretreated for 40 min with rapamycin (100 ng/mL) before cells were stimulated with TNF-α (100 ng/mL). The phosphorylations of (A) mTOR and (B) p70S6K were detected by immunoblot analysis, using antibodies phosphospecific for mTOR or p70S6K. (C) Inhibition of TNF-α-induced production of IL-6 and IL-8 by naringin. After a 1-day of starvation, the cells were pretreated with naringin at the indicated concentrations (μM) for 30 min before stimulation with or without TNF-α (100 ng/mL) for 24 h. Medium was collected and IL-6 and -8 concentration determined by ELISA. $^{**}p < 0.05$ compared with TNF-α treatment.

ylation of mTOR and p70S6K was activated by TNF-α (Fig. 6A and B). The inhibition by rapamycin of p70S6K phosphorylation indicates that p70S6K activation depends on mTOR (Fig. 6B). Finally, TNF-α-induced mTOR and p70S6K phosphorylation was inhibited by naringin (Fig. 6A and B). These results suggest that naringin inhibits the PI3K/AKT/ mTOR/p70S6K pathway.

3.6 Naringin inhibits TNF-α-stimulated production of IL-6 and IL-8

The effect of naringin on the release of IL-6 and IL-8 by TNF-α into medium at 24 h was measured. The release of IL-6 and IL-8 was stimulated by TNF-α (Fig. 6C). Co-incubation of TNF-αwith naringin reduced TNF-α-mediatedIL-6 and IL-8 production (Fig. 6C).

4 Discussion

Naringin, one of the most abundant flavonoids in citrus fruits, is present in peels and rinds, and has been shown to have protective effects in animal models of cardiovascular disease [23, 27, 28]. However, the exact molecular mechanisms underlying the MMP-9 regulation that results from naringin treatment remain to be identified. Our aim was to investigate the mechanism of the anti-atherogenic response of naringin against VSMC treated with TNF-α.

Our data showed that naringin treatment (less than 25 μM concentration) had no effect on TNF-α-stimulated VSMC proliferation. The results of thymidine uptake as an index of DNA synthesis in VSMC after naringin treatment indicated that there is no cessation of DNA synthesis. However, when naringin concentration was increased to 75 μM, cell death was observed. Our observation in this experiment is in general agreement with a previous report showing that naringin inhibited cell viability and cell proliferation in several cancer cell lines [19–21]. Because anti-atherogenic effects need not be limited to anti-proliferation, the effects of naringin on VSMC invasion and migration were further investigated. Dose-dependent inhibition by naringin of the invasion and migration of VSMC was observed. In previous *in vivo* experiments, TNF-α induced expression of a number of genes thought to be involved in regulating migration of VSMC, including genes encoding IL-6 and IL-8 [29, 30]. Elevated levels of IL-6 and IL-8 were found to be associated with migration and invasion [29, 30]. Here, we found that the addition of naringin inhibited the production of IL-6 and IL-8 induced by TNF-α. Our results suggest that treatment with naringin may contribute to the inhibition of the invasion and migration potential of VSMC.

MMPs are capable of degrading all components of the ECM and play key roles in normal physiological and pathological processes, such as wound healing and vascular remodeling [1–3]. MMP-9 production is increased after vascular injury, whereas in normal arteries it is not expressed [3, 31, 32]. It has generally been concluded that macrophage and VSMC are the major source of MMP-9 [31, 33, 34], and its expression can be induced by cytokine TNF-α [4–6, 31]. There are several lines of evidence that MMP-9 plays pivotal roles in ECM degradation, and in the invasion and migration of VSMC [2, 3, 7]. Increased evidence suggests that the expression of MMP-9 plays important roles in the pathogenesis of atherosclerosis and restenosis after vascular injury [7, 8, 35, 36]. Previous findings showed that TNF-α markedly induced MMP-9 expression in VSMC [4–6, 16]. In the present study, zymographic and immunoblot analyses showed that TNF-α-stimulated MMP-9 expression in VSMC was inhibited by naringin treatment in a dose-dependent manner. Under the same experimental conditions, treatment with naringin had no effect on the expression of MMP-2. The results indicate that inhibition of MMP-9 expression by naringin may have contributed to suppression of the invasion and migration of VSMC through ECM degradation.

Previous studies in our laboratory, as well as others, have demonstrated that TNF-α induced transcriptional MMP-9 promoter activity in several cell lines through AP-1 and NF-κB [13–16]. Consistent with the zymography and immunoblot analyses, our data from this study showed that MMP-9 promoter activity is effectively suppressed by naringin. Finally, we attempted to determine whether the decreased binding activities of AP-1 and NF-κB could account for the naringin-induced decrease in MMP-9 expression in VSMC. Using consensus AP-1 and NF-κB probes, a marked decrease was observed in both AP-1 and NF-κB binding activities in response to TNF-α in VSMC following treatment with naringin. A number of reports have appeared on the inhibition of cell invasion and MMP expression by flavonoids [26, 31, 37, 38]. Although the effect of flavonoids on the functions of MMP expression in VSMC has been analyzed [26, 31, 37], the molecular and cellular mechanisms underlying the inhibition of MMP expression by naringin in VSMC have not been examined. To our knowledge, this is the first systematic study demonstrating the inhibition of AP-1 and NF-κB binding activities by naringin in TNF-α-induced MMP-9 expression.

Studies of signal transduction pathways that regulate MMP-9 expression have demonstrated that the activation of the MAPKs, ERK1/2, p38, and PI3K/AKT is critical for the increased expression of MMP-9 in response to TNF-α (6, 16–18). It is known that many flavonoids suppress the phosphorylation of MAPKs, such as ERK1/2, p38, and JNK. However, in the present study, naringin did not inhibit the TNF-α-induced phosphorylation of ERK1/2, p38, and JNK, which led us to predict that different mechanisms underlie the suppression of TNF-α-induced migration of VSMC by naringin. It has been previously reported that TNF-α is found in atherosclerotic lesions and can have effects on VSMC including the stimulation of MMP-9 expression *via* the PI3K/AKT pathway [17, 18]. MMP-9 expression and migration have been shown to be mediated through activation of the PI3K/Akt signaling pathways [17, 18]. We attempted to determine the effect of naringin on TNF-α-induced PI3K/AKT/mTOR/p70S6K pathway in VSMC. Naringin treatment inhibited PI3K/AKT/mTOR/p70S6K pathway in TNF-α-stimulated VSMC. To our knowledge, this is the first study demonstrating the inhibition of PI3K/AKT/mTOR/p70S6K pathway by naringin in TNF-α-induced VSMC. Our observation in this experiment differs from a previous report that naringin (more than 100 μM concentration) inhibited phosphorylation of MAPKs, such as ERK1/2, p38, and JNK [39]. These results may explain the concentration-dependent differences in the inhibition of phosphorylation of MAPKs in cells following naringin treatment. However, a limitation of our study is that the direct inhibition mechanism by naringin between AKT and MMP-9 in TNF-α-induced VSMC has not been clarified. The exact mechanism needs to be elucidated.

Previous study showed that ERK1/2 was a main factor in TNF-α-induced MMP-9 expression in VSMC obtained from human [16]. The result from present study indicated that naringin completely inhibited TNF-α-induced MMP-9 expression in rat VSMC. However, naringin treatment did not show any effect on ERK activation. Considering these findings, this may explain the cell type species-differences in MMP-9 inhibition by naringin in low concentration on VSMC.

Orally administered naringin was hydrolyzed by enterobacteria to aglycones such as naringenin before being absorbed [40]. Previous studies showed that naringin and naringenin (less than 50 μM) treatment had no effect on cell viability in several cell lines, although there were differences of the inhibition of cell proliferation between naringin and naringenin (more than 100 μM) treatment in different cell lines [41–43]. We used the concentration of naringin at 25 μM in this experiment. We observed almost same effects on MMP-9 expression, invasion, migration, and AKT phosphorylation between naringin and naringenin treatment in TNF-α induced VSMC. These results show that treatment of naringin and naringenin in low concentration had no differences on VSMC invasion, migration, and MMP-9 expression in response to TNF-α.

Pharmacokinetic study has suggested that plasma concentration of naringin was 7.3 μM, when 472 μM of naringin is administered in human [41, 44]. The plasma concentration of the naringin was relatively high after ingestion of orange or grapefruit juice [44]. Naringin can accumulate in plasma after long-term intake, and they are distributed in tissues other than plasma [41]. Because of the limitation in detective analytical methods, distribution and accumulation of naringin in other tissues cannot be investigated [41, 44]. Thus, we suggest that benefit health effects could ensue in individuals consuming orange or grapefruit products regularly if naringin actually has biological significances *in vivo*.

It is notable that the inhibitory effects on migration and invasion were significant when naringin was used at 10–25 μM concentrations, with this level of naringin considered to have no toxicity. The present study provides important new insights into the molecular mechanisms of the effects of naringin on VSMC. First, naringin reduces cell migration and invasion in response to TNF-α. In addition, naringin potently inhibits TNF-α-induced MMP-9 expression by suppressing NF-κB and AP-1 binding activities. Furthermore, we demonstrated that naringin suppresses the TNF-α-induced PI3K/AKT/mTOR/p70S6K pathway. The findings of the present study may, in part, explain the preventive effects of naringin on cardiovascular diseases and atherosclerosis.

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