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Wogonin suppresses TNF-α-induced MMP-9 expression by blocking the NF-κB activation via MAPK signaling pathways in human aortic smooth muscle cells

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Abstract

Matrix metalloproteinase-9 (MMP-9) plays a major role in the pathogenesis of atherosclerosis and restenosis by regulating both migration and proliferation of vascular smooth muscle cells (VSMC) after an arterial injury. In this study, we examined the inhibitory effect of three major flavonoids in Scutellariae Radix, baicalin, baicalein, and wogonin, on TNF-α-induced MMP-9 expression in human aortic smooth muscle cells (HASMC). Wogonin, but not baicalin and baicalein, significantly and selectively suppressed TNF-α-induced MMP-9 expression in HASMC. Reporter gene, electrophoretic mobility shift, and Western blotting assays showed that wogonin inhibits MMP-9 gene transcriptional activity by blocking the activation of NF-κB via MAPK signaling pathways. Moreover, the Matrigel migration assay showed that wogonin reduced TNF-α-induced HASMC migration. These results suggest that wogonin effectively suppresses TNF-α-induced HASMC migration through the selective inhibition of MMP-9 expression and represents a potential agent for the prevention of vascular disorders related to the migration of VSMC.

Keywords: Wogonin; Scutellariae Radix; Vascular smooth muscle cells; Migration; TNF-α; MMP-9

The proliferation and migration of VSMC play a major role in the development and progression of many cardiovascular diseases, including atherosclerosis, and VSMC is the principal cell type in both atherosclerotic and restenotic lesions [1]. During the early stages of atherosclerosis or arterial wall injury, VSMC migrates into the intimal layer of arterial wall in response to platelet activation, thrombin generation, and the release of various growth factors and cytokines, causing intimal thickening [1–3]. Particularly, the cytokine TNF-α is secreted by VSMC in the neointima as well as by macrophages accumulated in atherosclerotic lesions and it markedly induces proliferation and migration of VSMC [4,5].

In addition, the degradation of extracellular matrix (ECM) which exert, biochemical and mechanical barriers to VSMC movement has been shown to be an important biological process in the proliferation and migration of VSMC [6,7]. ECM degradation and remodeling require the action of extracellular proteinases, among which the MMPs have been shown to play an essential role [8]. Recent reports based on an in vivo study concluded that type IV collagenases or gelatinases (MMP-2 and -9) are critical for the development of arterial lesions via its regulation of both VSMC migration and proliferation [9,10]. The synthesis and secretion of MMP-9 can be stimulated by a variety of stimuli including growth factors and

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cytokines [11,12]. On the basis of reports from several different laboratories, it has been generally concluded that the basal levels of MMP-9 in VSMC are usually low, and that its expression can be induced by treatment TNF-α via the activation of NF-κB and AP-1 [13,14].

Scutellariae Radix, the root of *Scutellaria baicalensis* Georgi, is a medicinal herb widely used for the treatment of various inflammatory diseases, hepatitis, hypertension, tumors, and fever in East Asian countries such as Korea, Taiwan, Japan, and China [15]. Scutellariae Radix contains a large number of flavonoids including baicalin, baicalein, and wogonin and the flavonoids have been known to be the major bioactive constituents of Scutellariae Radix. They have been shown to exert free radical scavenging [16], anti-inflammatory [17], antiviral [18], neuroprotective [19], anti-angiogenesis [20], and anticancer [21] activities. They also inhibited lipopolysaccharide (LPS)- or TPA-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expressions. In addition, it has been reported that wogonin has the therapeutic potential for the treatment of atherosclerosis and restenosis based on its antiproliferative [22], antithrombotic [23], anti-inflammatory [24], and anti-adhesion properties [25]. However, little information is available about their potentials on the migration of VSMC and the regulation of TNF-α-induced gene expressions.

In the present study, three major flavonoids in Scutellariae Radix, baicalin, baicalein, and wogonin, were examined for their potentials on TNF-α-induced MMP-9 expression in HASMC. Here we provide evidence showing that wogonin, but not baicalin and baicalein, suppresses TNF-α-induced MMP-9 expression by blocking the NF-κB activation via MAPK signaling pathways and the suppression of MMP-9 expression is correlated well with the inhibition of HASMC migration by wogonin.

Materials and methods

Materials. All chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Wogonin was obtained from Wako Pure Chemicals (Osaka, Japan). Baicalin, baicalein, and wogonin were dissolved in dimethyl sulfoxide (DMSO) and the maximum concentration of DMSO was 0.1%. Recombinant human TNF-α was obtained from R&D Systems (Boston, MA). Polyclonal antibodies to MAPK family and phospho-MAPK family were purchased from Cell Signaling Technologies (Beverly, MA). Polyclonal NF-κB (p65) and phospho-c-jun antibodies were purchased from Santa Cruz Biotechnology (California, USA). [γ^{32}-P]ATP was obtained from Amersham (Buckinghamshire, UK). Cell culture reagents were purchased from Gibco-BRL (Rockville, MD).

Cell cultures. HASMC were purchased from Bio-Whittaker (California, USA). HASMC were cultured in DMEM (Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) and 5% CO_2 at 37 °C. For all experiments, early passage HASMCs were grown to 80–90% confluence and made quiescent by serum starvation (0.1% FBS) for at least 24 h.

Cell viability assay. The cytotoxic effect of wogonin on HASMC was investigated using a commercially available proliferation kit (XTT II, Boehringer Mannheim, Mannheim, Germany). Briefly, the cells were plated in 96-well culture plate at a density of 1×10^4 cells/well in DMEM culture medium and allowed to attach for 24 h. After incubation, the medium was discarded and replaced with 100 μl of new medium containing various concentrations of wogonin. After 24 h of culture, 50 μl XTT test solution prepared by mixing 5 ml XTT labeling reagent and 100 μl of electron coupling reagent was added to each well. The optical density was read at 490 nm in an ELISA plate reader after 4 h of incubation with XTT test solution in a 37 °C and 5% CO_2 incubator.

Gelatin zymography assay. Conditioned medium was electrophoresed in a polyacrylamide gel containing 0.1% (w/v) gelatin. The gel was then washed at room temperature for 30 min with 2.5% Triton X-100 and subsequently incubated at 37 °C for 24 h in a buffer containing 10 mM $CaCl_2$, 0.01% NaN_3, and 50 mM Tris–HCl (pH 7.5). The gel was stained with 0.2% Coomassie brilliant blue and photographed on a light box. Proteolysis was detected as a white zone in a dark blue field.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted by the RNAzol B reagent (Tel-test, Friendswood, TX, USA) according to the manufacturer's instructions. For RT-PCR, a cDNA was synthesized from 1 μg of total RNA using AMV RNA PCR Kit (Takara, Japan) according to the manufacturer's protocol. The cDNA was amplified by PCR with the following primers: MMP-9 (537 bp), 5′-CGGAGCACGGAGACGGGTAT-3′ (sense) and 5′-TGAAGGGG AAGACGCACAGC-3′ (antisense); β-actin (247 bp), 5′-CAAGAGAT GGCCACGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCCTGTC GGCA-3′ (antisense). PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

Transient transfection and luciferase reporter assay. A 710 bp fragment from the 5′-promoter region of the MMP-9 gene was cloned [14]. A 710 bp fragment at the 5′-flanking region of the human MMP-9 gene was amplified by PCR using specific primers from the human MMP-9 gene (GenBank Accession No. D10051): 5′-ACATTTGCCCGAGCTCCTGAAG (forward/*Sac* I) and 5′-AGGGGCTGCCAGAAGCTTATGGT (reverse/*Hin*dIII). 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 *Sac*I/*Hin*dIII site of the pGL2-Basic vector (pGL2-MMP-9WT). PCR products (fragment of MMP-9 promoter) were confirmed by their size, as determined by electrophoresis, and by DNA sequencing. Cells were plated onto 6-well plates at a density of 2×10^5 cells/well and grown overnight. Cells were cotransfected with 1 μg MMP-9 promoter-luciferase reporter constructs and 0.5 μg of the pCMV-β-galactosidase reporter plasmid for 5 h using Lipofectamine reagent (Invitrogen, San Diego, CA, USA). After transfection, the cells were cultured in 10% FBS medium and incubated with drugs for 24 h. Luciferase and β-galactosidase activities were assayed by using the luciferase and β-galactosidase enzyme assay system (Promega). Luciferase activity was normalized with the β-galactosidase activity in the cell lysate and expressed as an average of three independent experiments.

Electrophoretic mobility shift assay (EMSA). The nuclear extract of cells was prepared as described below. Cells were washed with cold PBS and suspended in 0.4 ml of lysis buffer containing 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin. The cells were allowed to swell on ice for 15 min, and then 25 μl of 10% Nonidet P-40 was added. The tube was vigorously vortexed for 10 s, and the homogenate centrifuged at 4 °C for 2 min at 13,000 rpm. The nuclear pellet was resuspended in 50 μl of ice-cold nuclear extraction buffer containing 20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin. The tube was incubated on ice for 15 min with intermittent mixing. The nuclear extract was then centrifuged at 4 °C for 5 min at 13,000 rpm and the supernatant was either used immediately or stored at −70 °C for later use. The protein content was measured using the Bio-Rad protein assay. EMSA was performed using a gel shift assay system kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, double-stranded oligonucleotides containing the consensus sequences for AP-1-1 (5′-TGACCCCTGAGTCAGCACTT-3′) and NF-κB (5′-CC AGTGGAATTCCCCAG-3′) were end-labeled with [γ-^{32}-P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase and used as probes for EMSA. Competition was performed using either the unlabeled AP-1-1 or

NF-κB oligonucleotides. Nuclear extract proteins (2 μg) were preincubated with the gel shift binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl (pH 7.5), and 0.05 mg/ml poly deoxyinosine–deoxycytosine) for 10 min, then incubated with the labeled probe for 20 min at room temperature. Each sample was electrophoresed in a 4% nondenaturing polyacrylamide gel in 0.5 × TBE buffer at 250 Volt for 20 min. The gel was dried and exposed to X-ray film overnight.

Western blot analysis. HASMC were treated with various concentrations of wogonin in the presence of 100 ng/ml TNF-α. Cellular lysates were prepared in a lysis buffer containing 50 mM Tris–HCl (pH 7.5), 2 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1% NP-40. The cells were disrupted and extracted at 4 °C for 30 min. After centrifugation at 13,000 rpm for 15 min, the supernatant was obtained as the cell lysate. Protein concentrations were measured using the Bio-Rad protein assay. To determine the activations of NF-κB and AP-1, nuclear extracts of cells were isolated by the protocol of electrophoretic mobility shift assay (EMSA). Aliquots of cellular proteins (30 μg/lane) were electrophoresed on 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to an Immobilon-P-membrane (Millipore, USA). The membrane was allowed to react with a specific antibody and detection of specific proteins was carried out by enhanced chemiluminescence following the manufacturer's instructions. Loading differences were normalized using polyclonal β-actin antibodies.

Migration assay. Matrigel-coated filter inserts (8 μm pore size) that fit into 24-well migration chambers were obtained from Becton–Dickinson (New Jersey, USA). HASMC to be tested for migration were detached from the tissue culture plates, washed, and resuspended in conditioned medium (2×10^4 cells/well), then added to the upper compartment of the migration chamber in the presence or absence of drugs. Conditioned medium (500 μl) was added to the lower compartment of the migration chamber. The chambers were incubated at 37 °C for 24 h in 5% CO_2. After incubation, the filter inserts were removed from the wells and the cells on the upper side of the filter were removed using cotton swabs. The filters were fixed, stained, and mounted according to the manufacturer's instructions (Becton–Dickinson). The cells that migrated through the Matrigel and were located on the underside of the filter were counted. The microscopic photographs of migrated cells were kindly produced by M.S. Hyo Gwon Im (Dept. of Food Science and Technology, Keimyung Univ., Daegu, Republic of Korea). Three to five chambers were used per condition. The values obtained were calculated by averaging the total number of cells from three filters.

Statistical analysis. The results are expressed as means ± SE and differences between means for two groups were determined by unpaired Student's *t*-test. The minimum significance level was set at *P* value of $\leqslant 0.05$ for all analysis. All experiments were performed at least three times.

Results

Wogonin, but not baicalin and baicalein, suppresses TNF-α-induced MMP-9 expression in HASMC

Prior to the investigation into the pharmacological potential of three major flavonoids in Scutellariae Radix, baicalin, baicalein, and wogonin, on TNF-α-induced MMP-9 expression in HASMC, we first determined the dose dependence of the cytotoxic effects of the flavonoids in HASMC by means of an XTT assay. Baicalin at concentrations lower than 200 μM had little cytotoxic effect on the cells and baicalein and wogonin showed lower than 20% decrease in cell viability at 200 and 50 μM, respectively (Fig. 1A). We next used a gelatin zymography assay to investigate the inhibitory effect of the flavonoids on TNF-α-induced MMP-9 secretion. The media from control cells contained very weak proteolytic activity at 92 kDa, corresponding to MMP-9 and high proteolytic activity at 72 kDa, corresponding to MMP-2. Treatment with TNF-α (100 ng/ml) induced the level of such a band of proteolytic MMP-9 secretion. The induction of MMP-9 secretion by TNF-α was dramatically inhibited in the presence of wogonin in a dose-dependent manner in the concentration range

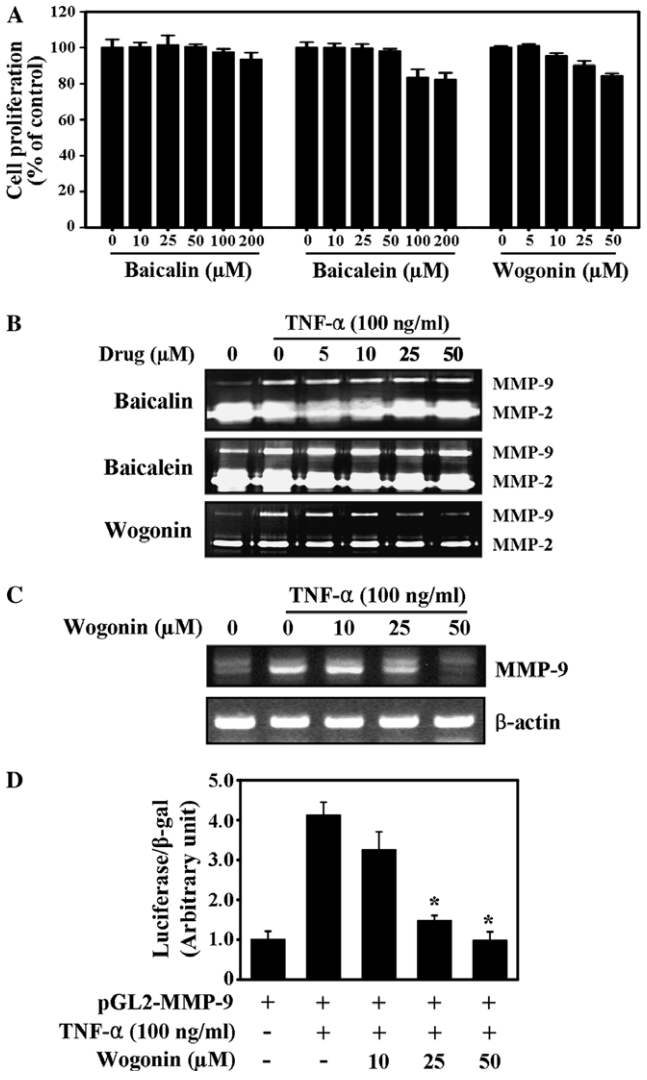

Fig. 1. Effects of baicalin, baicalein, and wogonin on the growth of HASMC and TNF-α-induced MMP-9 expression. Cells were treated with the indicated concentrations of drugs for 24 h. Cell viability was determined by an XTT assay (A). Cells were treated with the indicated concentrations of drugs in the presence of TNF-α (100 ng/ml) for 24 h. The conditioned medium was prepared and used for gelatin zymorgraphy (B). The MMP-9 mRNA levels were measured by RT-PCR. β-Actin was used as an internal control (C). pMMP-9WT-luciferase vector was cotransfected with pCMV-β-galactosidase vector into the cells. After 5 hours transfection, the cells were treated with the indicated concentrations of wogonin in the presence of TNF-α (100 ng/ml) (D). Luciferase activities were normalized to β-galactosidase activity. Each value represents the means ± SE of three independent experiments and is expressed relative to a control; $^*P < 0.001$ vs TNF-α.

of 5–50 μM, however, baicalin and baicalein did not show any inhibitory effect on the induced MMP-9 secretion (Fig. 1B). On the other hand, the level of MMP-2 was not altered by TNF-α and none of the flavonoids affected the MMP-2 secretion (Fig. 1B).

Wogonin suppresses TNF-α-induced MMP-9 secretion through inhibition of its transcriptional activity in HASMC

To determine whether the inhibition of MMP-9 secretion by wogonin was due to a decreased level of transcription, we performed RT-PCR and promoter assay using transiently transfected cells with a luciferase reporter gene linked to the MMP-9 promoter sequence. Treatment of cells with wogonin decreased the levels of TNF-α-stimulated MMP-9 mRNA expression (Fig. 1C). As shown in Fig. 1D, luciferase activity was increased up to 4-fold in cells treated with TNF-α as compared with untreated cells. Treatment of cells with wogonin decreased TNF-α-stimulated luciferase activity in a dose-dependent manner. These results show that wogonin suppresses TNF-α-induced MMP-9 secretion through inhibition of its transcriptional activity in HASMC.

Wogonin inhibits TNF-α-induced MMP-9 expression by blocking the NF-κB activation

It has recently been reported that the MMP-9 promoter contains *cis*-acting regulatory elements for transcription factors that include two AP-1 sites (located at −79 and −533 bp) and an NF-κB site (located at −600 bp), and the AP-1 and the NF-κB elements are centrally involved in the induction of the MMP-9 gene by TNF-α in VSMC [14,12,26]. To investigate which of these transcription factors are involved in the inhibition of the MMP-9 transcription by wogonin in HASMC, we examined the inhibitory effect of wogonin on the binding of AP-1 and NF-κB isolated from TNF-α-induced HASMC to oligonucleotides that contain the sequence for the AP-1 and NF-κB binding sites from the MMP-9 promoter using EMSA. HASMC were incubated with different concentrations of wogonin in the presence of TNF-α for 30 min, and nuclear extracts were prepared and tested by EMSA. As shown in Fig. 2A, wogonin at a concentration of 50 μM completely inhibited the TNF-α-induced binding activity of NF-κB, whereas it did not affect on the TNF-α-induced binding activity of AP-1.

TNF-α has been shown to induce the phosphorylation of p65, a major subunit of NF-κB leading to their translocations to nucleus which are required for the transcriptional activities [27]. Therefore, we examined the effect of wogonin on the TNF-α-stimulated nuclear translocation of p65 subunit by Western blot analysis. As shown in Fig. 2B, TNF-α induced the nuclear translocation of p65 and phosphorylation of c-Jun, a major subunit of AP-1, and wogonin inhibited the translocation of p65 in a dose-dependent manner. As expected, however, the phosphorylation level of c-Jun was not affected by wogonin. These data clearly show that wogonin regulates the transcriptional activation of MMP-9 through the inhibition of TNF-α-stimulated NF-κB activities.

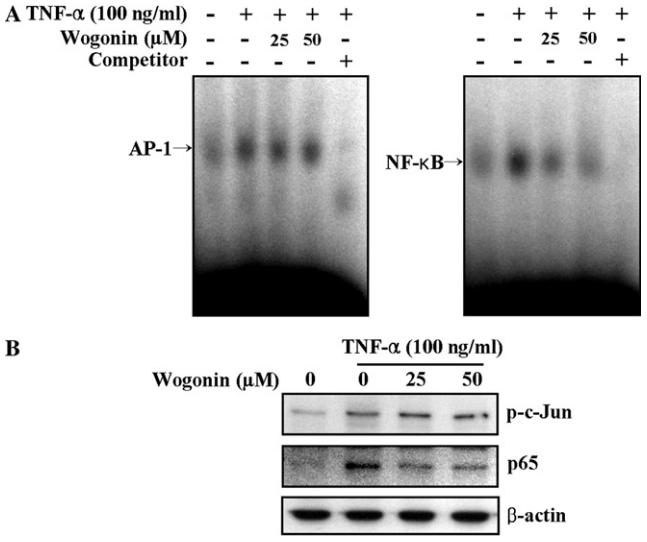

Fig. 2. Effects of wogonin on the TNF-α-induced AP-1 and NF-κB activations in HASMC. Cells were treated with the indicated concentrations of wogonin in the presence of TNF-α (100 ng/ml). Nuclear extracts were prepared and examined for AP-1 and NF-κB activations by EMSA. Competition was performed using an unlabeled AP-1 or NF-κB double-stranded oligonucleotides (A). The nuclear extracts were also examined for phospho-c-Jun and p65 protein expressions by Western blotting. β-Actin was used as an internal control (B).

MAPK signaling pathways are involved in the inhibition of TNF-α-induced MMP-9 expression by wogonin in HASMC

MMP-9 gene expression can be activated via a number of signal transduction pathways including those involving ERK1/2, p38 MAPK, JNK, and PI3K/Akt, which are the upstream modulators of AP-1 or NF-κB [11,12,28,29]. The subsequent experiments were designed to elucidate which of these signal transduction pathways is involved in TNF-α-stimulated MMP-9 expression and wogonin inhibition of the MMP-9 expression in HASMC. First, the effects of specific kinase inhibitors on the expression of MMP-9 in TNF-α-induced HASMC were analyzed by zymography. TNF-α-induced MMP-9 secretion was inhibited by selective inhibitors of the ERK1/2 (U0126), p38 MAPK (SB203580), or JNK (SP600125) pathways, but not by inhibitors of the PI3K/AKT (Wortmanin) or PKC (Gö6976) pathways (Fig. 3A). Then we investigated whether wogonin inhibited MMP-9 secretion by blocking activation of the ERK1/2, p38 MAPK, or JNK pathways. TNF-α induced the phosphorylation of all of three members of the MAPKs as early as 5 min, with a maximal phosphorylation at 15 min (data not shown). Wogonin showed inhibitory effects on the phosphorylation of ERK1/2, p38 MAPK, and JNK pathways in a dose-dependent manner at 15 min after TNF-α treatment (Fig. 3B). These results

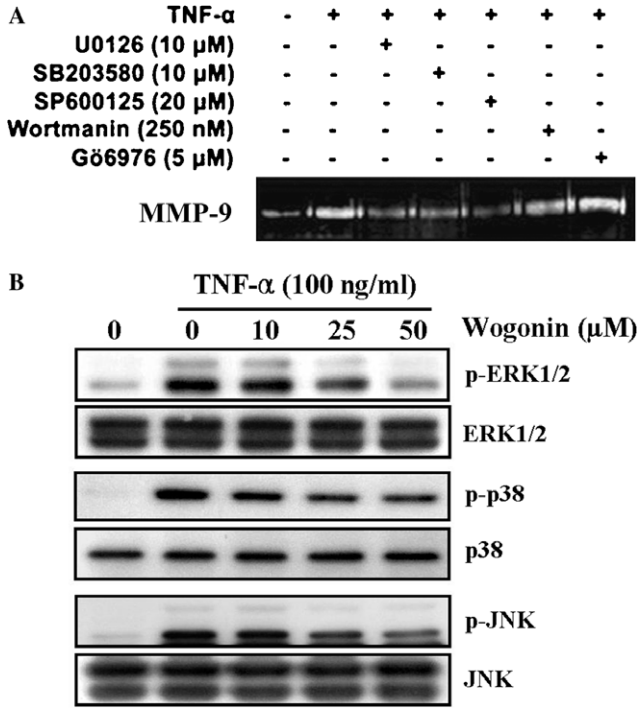

Fig. 3. Effects of wogonin on TNF-α-induced activation of MAPK signaling pathways in HASMC. Cells were stimulated with TNF-α (100 ng/ml) for 24 h in the presence or absence of each inhibitor, and MMP-9 secretion in the conditioned medium was determined by gelatin zymorgraphy (A). Cells were treated with TNF-α (100 ng/ml) for 15 min in the presence or absence of wogonin, and the phosphorylation levels of ERK1/2, p38 MAPK, and JNK were measured by Western blotting (B).

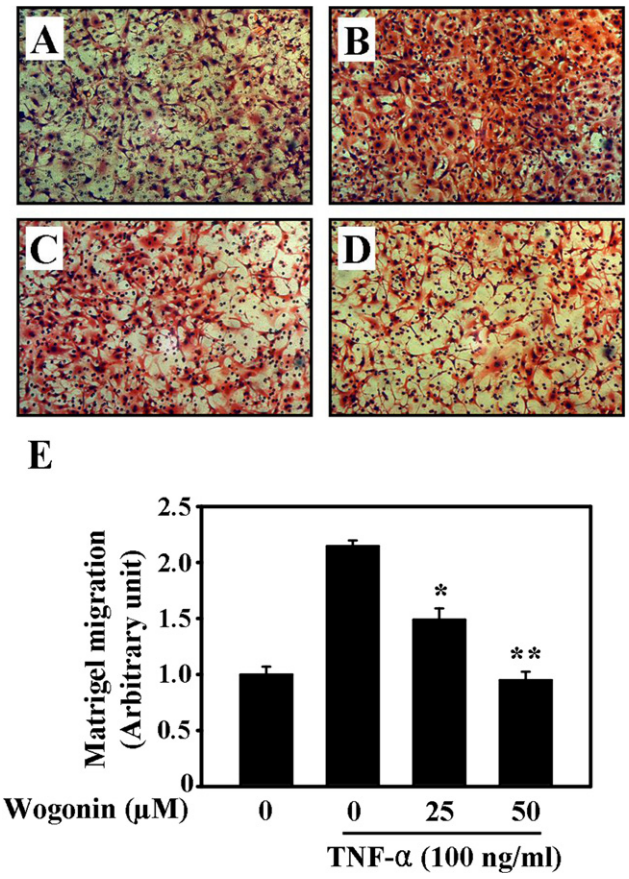

Fig. 4. Effect of wogonin on TNF-α-induced Matrigel migration of HASMC. A Matrigel migration assay was carried out with 25 and 50 μM wogonin in the presence of TNF-α (100 ng/ml). After 24 h incubation, cells on the bottom side of filter were fixed, stained, and counted. (A) control; (B) TNF-α alone; (C) TNF-α with wogonin (25 μM); (D) TNF-α with wogonin (50 μM). Each value represents the means ± SE of three independent experiments and is expressed relative to a control; $^{*}P < 0.01$ vs TNF-α, $^{**}P < 0.001$ vs TNF-α.

suggest that the specific inhibitions of MAPK signaling pathways are directly involved in the regulation of TNF-α-induced MMP-9 expression by wogonin in HASMC.

Wogonin inhibits TNF-α-induced HASMC migration in vitro

It has been reported that the up-regulation of MMP-9 expression contributes to migration of VSMC in vivo and in vitro [9,30]. Thus, we examined whether the migration of TNF-α-induced HASMC was decreased by wogonin, which previously showed a selective inhibition of TNF-α-induced MMP-9 expression. As shown in Fig. 4, the migration of HASMC was increased by treatment with TNF-α when compared with TNF-α-untreated control cells, as evidenced by a Matrigel migration assay. However, wogonin inhibited the TNF-α-induced HASMC migration in a dose-dependent manner.

Discussion

In the past several years, a number of studies have demonstrated that proliferation and migration of VSMC play a major role in the pathogenesis of atherosclerosis and restenosis after vascular injury [8,30] and MMPs, specifically MMP-2 and MMP-9, are important for SMC proliferation and migration into the intima [31]. Because MMP-2 and MMP-9 play pivotal roles in proliferation and migration of VSMC, the inhibitory effect on their expression is important for therapeutic experimental model of atherosclerosis.

Wogonin, 5,7-dihydroxy-8-methoxyflavone, is a flavonoid derived from Scutellariae Radix, a medicinal plant traditionally used in Oriental medicine. Wogonin has previously been shown to have the therapeutic potential for the treatment of atherosclerosis and restenosis. It has been reported that wogonin suppresses the proliferation of VSMC [22], the elevation of trypsin-induced plasminogen activator inhibitor-1 production in endothelial cells [23], and monocyte adhesion to endothelial cells by inhibiting 12-lipoxygenase activity in human platelets [24] and phorbol ester (PMA)-induced monocyte chemotactic protein-1 gene expression in endothelial cells [25]. However, the effect of wogonin on the gene expressions of MMP-2 and MMP-9, crucial factors for atherosclerosis and restenosis, in VSMC and the subsequent influence on VSMC migration are currently unknown. Thus, we further demonstrated here that wogonin suppresses the migration of VSMC

through inhibition of TNF-α-induced MMP-9 expression with its detailed molecular mechanisms, supporting the previous reports of its therapeutic potentials in atherosclerosis and restenosis.

Our results demonstrated that wogonin, but not baicalin and baicalein, inhibits the TNF-α-induced MMP-9 secretion through suppression of the transcriptional activity of MMP-9 gene in HASMC (Figs. 1 and 2). Baicalin and baicalein even at 200 μM, the highest concentration examined, did not show any inhibitory effect on the MMP-9 expression (data not shown). Interestingly, baicalin and baicalein, however, inhibited the enzymatic activity of the MMP-9 secreted from TNF-α-induced HASMC, but wogonin failed to inhibit its enzymatic activity (data not shown). Although all of these flavonoids share a similar chemical structure, there are differences in their relative efficacy in various assay systems, as well as in their pharmacokinetics [32,33]. In the previous report, Lai et al. [33] also suggested that wogonin is relatively nonpolar and thus more permeable through enterocytes. From this suggestion, we predict that difference in uptake levels of the flavonoids by HASMC caused by the difference of their lipophilicity might be the reasons for the issue of selectivity and potency.

To determine the inhibitory effect of wogonin on MMP-9 gene transcription through suppression of transcription factor activity, we carried out an EMSA. Wogonin blocked the activation of NF-κB, but not AP-1, by suppressing the interaction of NF-κB proteins with oligonucleotides that contain the sequence for the NF-κB binding sites from the MMP-9 promoter (Fig. 2). On the other hand, previous studies showed that SP-1 site of MMP-9 promoter is also involved in the induction of its gene expression in response to TNF-α in tumor cell lines [34]. However, Moon et al. [12] reported that SP-1 binding activity is not stimulated by TNF-α in HASMC. These data clearly indicate that wogonin blocks TNF-α-induced MMP-9 production mainly through inhibition of NF-κB-mediated MMP-9 induction.

Several studies have identified signal transduction pathways that are involved in regulation of MMP-9 expression in tumor cells [35,36], endothelial cells [37], keratinocytes [38], and VSMC [11,12]. In this study, the effects of various kinase inhibitors on the expression of MMP-9 were investigated and we found that TNF-α-induced MMP-9 activation was decreased by ERK1/2, p38 MAPK, or JNK inhibitors but not PI3K (wortmannin), or PKC-α and -β inhibitor (Gö6976). Although our observation in this experiment is in general agreement with previous reports showing that TNF-α-induced MMP-9 activaition was inhibited by U0126, SB203580, and SP600125 in VSMC [11,12], other findings indicate that activation of PKC plays a critical role in the MMP-9 expression in various cell types when they are induced by other stimulators such as PMA [39], IL-1β [40], or fibroblast growth factor-2 [41]. In addition, higher dose of TNF-α appears to be required to induce MMP-9 expression sufficiently in HASMC than in other cells such as human bronchial epithelial cells [42], human cholangiocarcinoma CCKS1 [43]. Considering these findings, this may explain the cell type-specific or stimulation factor-specific difference in the regulation of MMP-9 expression. Here we also identified the signal pathway-mediated regulation of the MMP-9 gene in TNF-α-induced HASMC in response to the treatment with wogonin. The data here show that all of 3 members of the MAPKs are major pathways in the wogonin-mediated inhibition of MMP-9 expression in TNF-α-induced HASMC (Fig. 3B). These findings suggest that the MAPK pathways appear to be required for TNF-α-induced MMP-9 expression in HASMC and wogonin regulates TNF-α-stimulated MMP-9 expression by suppressing the MAPK pathways.

The data obtained from the Matrigel migration assay show that the selective induction of MMP-9 expression by TNF-α causes the stimulation of HASMC migration, however, wogonin significantly inhibits the TNF-α-induced migration potential of HASMC (Fig. 4), suggesting that the inhibition of HASMC migration by wogonin is correlated well with the inhibition of MMP-9 expression.

Biological activities of flavonoids depend on their absorption. The chemical structures and physicochemical properties of flavonoids determined their rates and extents of intestinal absorption and the nature of the metabolites circulating in the plasma [33]. It has been reported that wogonin is absorbed by small intestine in a greater extent and the apparent elimination half-life of wogonin metabolites is longer than those of baicalin and baicalein metabolites in human [33]. Based on these reports, wogonin could be expected as a reliable flavonoid which possibly exerts the antimigrative potential in VSMC in vivo. Therefore, these reports with the new beneficial effect of wogonin in the present study may expand future researches on its regulation of VSMC proliferation and migration in vivo, as well as the detailed mechanisms.

In conclusion, as illustrated in Supplementary figure, wogonin suppresses TNF-α-induced MMP-9 expression in HASMC by inhibiting the activation of NF-κB via the MAPK signaling pathways. This is the first study showing that wogonin effectively suppresses TNF-α-stimulated migration of HASMC through inhibition of MMP-9 expression, and suggesting that wogonin is proposed to a potential candidate for the prevention of vascular disorders related to the migration of VSMC.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.006.

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