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3,4,5-Trihydroxybenzaldehyde From *Geum japonicum* Has Dual Inhibitory Effect on Matrix Metalloproteinase 9; Inhibition of Gelatinoytic Activity as well as MMP-9 Expression in TNF-α Induced HASMC

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ABSTRACT

The matrix metalloproteinases (MMP-9 and MMP-2) production and smooth muscle cell (SMC) migration may play key roles in the pathogenesis of atherosclerotic lesions. In particular, the cancer cell invasion and SMC migration through vascular wall were shown to be directly associated with inducible MMP-9 expression. Previously, 3,4,5-trihydoroxybenzaldehyde (THBA) was purified from *Geum japonicum* and we demonstrated a direct inhibition effect of THBA on MMP-9 and MMP-2 activity in the supernatants of TNF-α-induced HASMCs. In addition, MMP-9 expression and migration was suppressed by THBA in the TNF-α-induced HASMCs. In this study, we also investigated whether TNF-α-induced MMP-9 expressions are involved with migrations of HASMCs by using cell signal inhibitors and MMP-9 inhibitors. An RT-PCR and luciferase-tagged promoter analysis revealed that THBA inhibits the transcription of MMP-9 mRNA. Moreover, an electrophoretic mobility shift assay (EMSA) exhibited that THBA also suppressed DNA binding of nuclear factor (NF)-κB and activator protein (AP)-1 transcription factors. Furthermore, Western blot analysis indicated TNF-α-induced phosphorylation of extracellular signal regulated kinase 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinase (JNK) were inhibited by THBA. Taken together, we suggest that THBA has inhibition effect to the migrations as well as MMP-2 and MMP-9 activities in HASMCs. Especially gelatinolytic activity was controlled by enzymatic inhibition of MMP-2 and MMP-9, and also down-regulated MMP-9 transcription via mitogen-activated protein kinase (MAPK) pathways in THBA treated HASMCs. J. Cell. Biochem. 105: 524–533, 2008.

KEY WORDS: THBA; 3,4,5-TRIHYDROXYBENZALDEHYDE; MATRIX METALLOPROTEINASE-9; MATRIX METALLOPROTEINASE-2; HUMAN AORTIC SMOOTH MUSCLE CELLS; TUMOR NECROSIS FACTOR-ALPHA; MITOGEN-ACTIVATED PROTEIN KINASE

The abnormal growth of vascular smooth muscle cells (VSMCs) in addition to the degradation and remodeling of extracellular matrix (ECM) proteins are important features of vascular disease, including atherosclerosis and restenosis after angioplasty [Ross, 1993]. A number of studies have indicated that matrix metalloproteinases (MMPs), specifically MMP-2 and MMP-9, are required for smooth muscle cell (SMC) proliferation and migration into the intima [Newby and Zaltsman, 2000]. An enhanced proteolytic activity in the vessel wall mediates the degradation of the ECM surrounding SMCs in response to injury [Newby and Zaltsman, 2000], and this a necessary process which allows the medial SMCs to migrate into the intimal space. In rat arterial injury model, MMP-9 is expressed within 6 h after injury in rat carotid arteries and persists to be expressed for up to 6 days, whereas MMP-2 activity is substantially increased 4 days after injury [Bendeck et al., 1994]. As a result of a knockout study, MMP-9 was found to be critical for the development of arterial lesions, because of its ability to regulate both vascular smooth muscle cells migration and proliferation [Cho

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and Reidy, 2002; Galis et al., 2002]. Although they have similar substrate specificities, MMP-2 and MMP-9 differ in their regulation of expression [Senior et al., 1991; Okada et al., 1992]. In several cell types, including smooth muscle cells, MMP-2 is constitutively expressed and not induced by cytokines or growth factors [Galis et al., 1994; Fabunmi et al., 1996]. In contrast, it has widely been concluded that the basal level of MMP-9 is usually low, and its expression can be induced by tumor necrosis factor-α (TNF-α), and not by the platelet-derived growth factor (PDGF) or thrombin [Galis et al., 1994; Fabunmi et al., 1996; Cho et al., 2000]. In the studies elucidating the signal transduction pathways involving the regulation of MMP-9 expression, it was demonstrated that the activation of the extracellular signal-regulated kinase 1/2 (ERK1/2), p38 kinase, c-Jun N-terminal kinase (JNK) and PI-3 kinase are important in the response to different stimulators and cell lines [Goetze et al., 1999; Cho et al., 2000; Goetze et al., 2001; Faisal Khan et al., 2002; Moon et al., 2004; Yao et al., 2004; Ohtsu et al., 2005]. Atherogenic lesions form during several pathological processes, which involve the accumulation of inflammatory cells as well as the release of cytokines [Abedi and Zachary, 1995]. Moreover, TNF-α is secreted by VSMC in the neointima after a balloon-injury as well as by macrophages [Tipping and Hancock, 1993; Clausell et al., 1995; Jovinge et al., 1997]. TNF-α induces the activation of ERK1/2, a key transducer of extracellular signals that promote cell growth and movement, which are critical for the initiation and progression of vascular lesions [Goetze et al., 1999; Goetze et al., 2001; Suh et al., 2006].

Several natural products have been used for vascular diseases [Heber, 2001], and some of these compounds have also been employed for the treatment of atherosclerosis [Yoshie et al., 2001; Kim et al., 2003]. *Geum japonicum* is an herbal plant which occurs in Asian countries and is used as diuretic. Recently it was reported that the extract of *G. japonicum* has angiogenic and anti-apoptotic effects [Cheung et al., 2007]. Several tannins purified from *G. japonicum* were found to have anti-cocagulative properties. As well triterpenes were found to have anti-HIV activities [Xu et al., 1996; Dong et al., 1998]. The newly purified compound, trihydoxybenzaldehyde (THBA), from *G. japonicum* in Korea was previously reported as an anti-oxidant [Kim et al., 2006] and in this article, we showed its inhibition effect on the MMP-2 and MMP-9, as well as migrations of HASMCs.

MATERIALS AND METHODS

MATERIALS

THBA was kindly provided by Dr. Jung Bong Kim, Department of Molecular Physiology and Biochemistry, National Institute of Agricultural Biotechnology (NIAB) of Korea [Kim et al., 2006]. THBA was dissolved in dimethyl sulfoxide (DMSO) (chemical structure was showed in Fig. 1A). Recombinant human TNF-α was obtained from R&D systems (Minneapolis, MN). The monoclonal antibodies to p-ERK1/2, p-p38 and p-SAPK/JNK were purchased from New England Biolabs (Beverly, MA). PI3K inhibitor, Wortmannin (#681675) and MAPK inhibitors including U0126 (#662005), SB203580 (#559389), SP600125 (#420119), cell permeable I-JIP (Inhibitor of JNK based on JNK interacting protein-1, #420116) and recombinant TIMP-1 (#PF019) were purchased from Calbiochem.

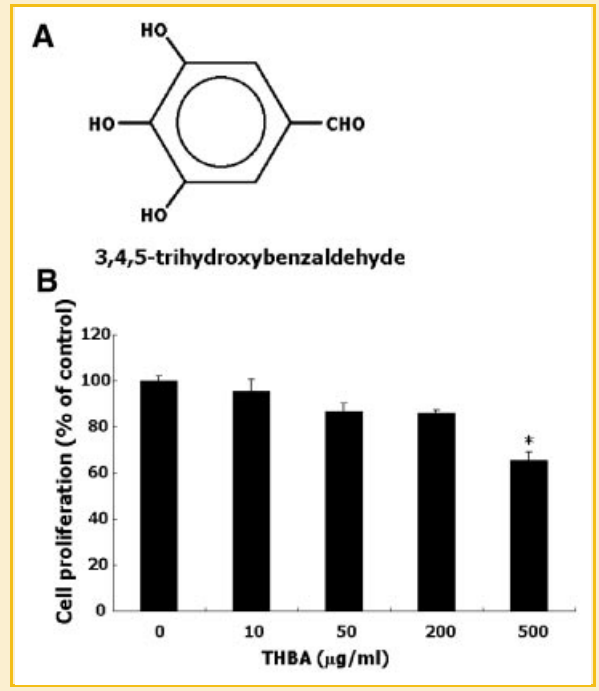

Fig. 1. The chemical structure of THBA and its effect on the viability of HASMC. A: The chemical structure of 3,4,5-Trihydroxybenzaldehyde (THBA) from *Geum japonicum* B: HASMC was plated onto a 96-well plate and grown to 70–80% confluence followed by replacement in each well with serum-free media containing different concentrations of THBA (0, 10, 50, 200, and 500 μg/ml). The XTT solution was added after 20 h. After 24 h, the optical density was measured at 490 nm. The results are expressed as the mean ± SE from three triplicate experiments. $^{*}P < 0.05$ compared with untreated control using Student's *t*-test.

CELL CULTURES

HASMC were purchased from Bio-Whittaker (San Diego, CA) and cultured in smooth muscle cell growth medium-2 containing 10% fetal bovine serum (FBS), 2 ng/ml human basic fibroblast growth factor, 0.5 ng/ml human epidermal growth factor, 50 mg/ml gentamicin, 50 mg/ml amphotericin-B, and 5 mg/ml bovine insulin. 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. The serum-free medium contained secreted proteins, such as MMP-9 the amount of secreted proteins in the conditioned media was estimated and quantified by cell numbers.

PROMOTER ASSAY

A 710 bp fragment from the 5′-promoter region of the MMP-9 gene was cloned. 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 (accession no. D10051): 5′-ACAT-TTGCCCGAGCTCCTGAAG (forward/*Sac*I) and 5′-AGGGGCTGCCA-GAAGCTTATGGT (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 the

PCR-amplified DNA of the MMP-9 promoter into the *Sac*I/*Hin*dIII site of the pGL2-Basic vector and named as the WT-MMP9pro [Moon et al., 2004]. PCR products (fragment of MMP-9 promoter) were confirmed by their size, via electrophoresis and DNA sequencing. HASMCs were plated onto 6-well plates at a density of 10^5 cells/well and grown overnight. Next, cells were cotransfected with 1 μg of MMP-9 promoter-luciferase reporter constructs and 1 μg of β-galactosidase reporter plasmid by the LipofecAMINE method (Invitrogen, San Diego, CA). Transfected cells were recovered in 10% FBS medium, serum starved, and then incubated with THBA in the presence or absence of TNF-α for 24 h. Luciferase activity and β-galactosidase activity were assayed via luciferase and β-galactosidase enzyme assay system (Promega, Madison, WI). Luciferase activity was normalized with the β-galactosidase activity in the cell lysate and calculated as an average of three independent experiments.

WESTERN BLOTTING

Quiescent HASMCs were treated in the presence or absence of TNF-α as indicated. To detect MMP-9 and GAPDH protein in Western blot analysis, the cells were homogenized in a sample buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% NaN_3, 100 mg/ml PMSF, 1 mg/ml aprotinin, and 1% Triton X-100. The protein concentrations were measured using the Bio-Rad protein assay, and the blotted membranes were incubated with MMP-9 (Serotec, Kidlington, Oxford, UK) and GAPDH antibodies (Chemicon, El Segundo, CA). The monoclonal antibodies and polyclonal antibodies of p-ERK1/2, p-SAPK/JNK, and p-p38 were purchased from New England Biolabs. Detection was performed using a secondary horseradish peroxidase-linked anti-mouse antibody and the ECL chemiluminescence system (Amersham, Arlington Heights, IL).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The nuclear extract of each cell was prepared as described in a previous reports [Chung et al., 2004]. EMSA were performed using a gel shift assay system kit (Promega) according to the manufacturer's instructions. Briefly, double-stranded oligonucleotides containing the consensus sequences for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and AP-1 (5′-CTGACCCCTGAGTCAGCACTT-3′) were end-labeled with [γ-^{32}P] ATP (3000 Ci/mmol; Amersham Pharmacia Biotech) using T4 polynucleotide kinase and used as probes for EMSA. Competition was performed using either the unlabeled NF-κB and AP-1 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 dithiothreitol, 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 V for 20 min. The gel was dried and subjected to autoradiography.

REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT-PCR)

To detect the expression of MMP-9 using RT-PCR, total RNA was prepared from HASMCs using the RNAzol B reagent (Tel-test, Friendswood, TX) according to the manufacturer's instructions. Samples were precipitated with Isopropanol, resuspended and treated with DNase I. For the RT-PCR, cDNA was synthesized from 1 μg of total RNA using the AMV RNA PCR Kit (Takara, Japan) according to the manufacturer's protocol. The cDNA was amplified with PCR using the following primers: MMP-9 (537 bp), 5′-CGGAGCACGGAGACGGGTAT-3′ (sense) and 5′-TGAAGGGGAAGACGCACAGC-3′ (antisense); β-actin (247 bp), 5′-CAAGAGATGGCCACGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCCTGTCGGCA-3′ (antisense); Timp-1 (527 bp), 5′-CCACCCCACCCACAGACG-3′ (sense) and 5′-CGCAGGGACTGCCAGGTG-3′ (antisense); Timp-2 (508), 5′-GCGCTCGGCCTCCTGCT-3′ (sense) and 5′-CTGTGACCCAGTCCATCC AGAGG-3′ (antisense). The PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

CELL VIABILITY ASSAY

The cytotoxic effect of the THBA on HASMCs was investigated using a commercially available proliferation kit (XTT II, Boehringer Mannheim, Mannheim, Germany). Briefly, the cells were plated onto 96-well culture plates at a density of 5×10^3 cells/well in culture medium and allowed to attach for 24 h. After incubation, the medium was discarded and replaced with 100 μl of serum-free medium containing various concentrations of THBA (0, 10, 50, 200, and 500 μg/ml). After an additional 24 h of culture, 50 μl of XTT reaction solution (sodium 3′-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and *N*-methyl dibenzopyrazine methyl sulfate mixed at a proportion of 50:1) was added to the wells. The optical density was read at 490 nm in an ELISA plate reader after incubating the plates for 4 h with XTT at 37°C and 5% CO_2 + 95% air. All determinations were confirmed using replication in at least three identical experiments and the data were expressed as the mean ± SE as a function of percent control.

INVASION ASSAYS

Matrigel invasion assay was performed as described previously [Chung et al., 2002]. Briefly, Matrigel-coated filter inserts (8 μm pore size) that fit into 24-well invasion chambers were obtained from BDbioscience (San Jose, CA). HASMCs (5×10^4 cells/well), which were to be tested for invasion, were detached from the tissue culture plates, washed and resuspended in conditioned medium (without-TNF-α, TNF-α-treated only or THBA (0, 20, 50, 100 μg/ml) + TNF-α treated medium), and then added to the upper compartment of the invasion chamber. Next, 500 μl of TNF-α treated medium was added to the lower compartment of the invasion chamber. The Matrigel invasion chambers were incubated at 37°C for 24 h in 5% CO_2. After incubation, the filter inserts were removed from the wells, followed by removal of the cells on the upper side of the filter using cotton swabs. The filters were fixed, mounted, and stained according to the manufacturer's instructions. The cells located on the underside of the filter, which invaded through the Matrigel, were counted. Three to five invasion chambers were used per condition. The values obtained were calculated by averaging the total number of cells from three filters.

GELATIN ZYMOGRAPHY ASSAY

Gelatin zymography was performed as described previously [Cha et al., 2003] with some modification. The culture supernatants of HASMCs treated with or without TNF-α (100 ng/ml) were resuspended in a sample buffer containing 62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.00625% (w/v) bromophenol blue, and were loaded without boiling in 7.5% acrylamide/bisacrylamide (29.2:0.8) separating gel containing 0.1% (w/v) gelatin. Electrophoresis was carried out at a constant voltage of 100 V. After electrophoresis, the gels were soaked in 0.25% Triton X-100 (twice for 30 min) at room temperature and rinsed in pure distilled water. To evaluate the inhibitory effect of THBA on the TNF-α induced MMP-9 expression, HASMCs were grown to sub-confluence and rinsed with PBS, followed by incubation in serum-free media with or without drug (THBA) in the presence of TNF-α for 24 h. Next, the conditioned media were collected and resolved in 10% polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were washed for 1 h in 2.5% (v/v) Triton X-100 to remove SDS, and then incubated for 24 h at 37°C in the incubation buffer to allow for the proteolysis of the gelatin substrate. The bands corresponding to activity were visualized by negative staining with Coomassie Brilliant blue R-250 (Bio-Rad) and the molecular weights were estimated by reference to prestained SDS–PAGE markers.

IMAGE ANALYSIS

The intensity of the bands obtained from zymogram studies and Western blot images was captured and estimated with Scion Image (Scion, MA).

RESULTS

CYTOTOXICITY OF THBA ON HASMCs

The cytotoxity of THBA on HASMCs was evaluated using XTT cell proliferation assay kit. The HASMCs (5×10^3 cells/well) were incubated for 24 h in 96-well microplates with various final concentrations of THBA (0, 10, 50, 200, and 500 μg/ml). As shown in Figure 1B, HASMCs treated with 500 μg/ml of THBA showed 65.6% growth ratio compared with non-treated (100%).

THBA INHIBITS MMP-2 AND MMP-9 ACTIVITY

It is well known that MMP-9 is a critical mediator of atherosclerosis and cancer metastasis [Cho and Reidy, 2002; Chung et al., 2004]. We first attempted to investigate the inhibitory effect of THBA on the enzymatic activity of MMP-9 from a series of cell lines. We used the supernatants containing MMP-2 and MMP-9 from TNF-α-stimulated HASMCs. In order to evaluate the inhibitory activity of THBA on MMP-9 or MMP-2 from the supernatants, which were electrophorized in gelatin SDS–PAGE. The MMP-9 and MMP-2 containing gel slices were incubated at various concentrations of THBA in incubation buffer for 24 h at 37°C and stained with Coomassie Brilliant blue. The MMP-2 and MMP-9 activities of the supernatants were decreased as a result of treatment with THBA at concentrations ranging from 20 to 500 μg/ml in a dose-dependent manner (Fig. 2A).

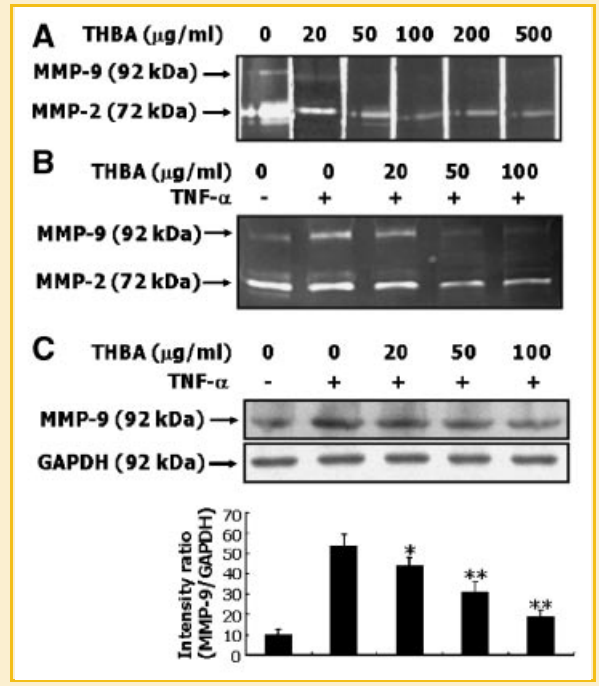

Fig. 2. THBA inhibits MMP-9 production as well as gelatinolytic activity in TNF-α-induced HASMC cultures. A: HASMC cultures incubated with 100 ng/ml of TNF-α in serum-free growth medium for 24 h and the supernatants were used for inhibition assay. Various concentrations of THBA (0, 20, 50, 100, 200, 500 μg/ml) were added to the incubation buffers and were incubated at 37°C for 24 h. CBB was used to stain the gelatin SDS–PAGE. We tested the in-vitro inhibition function 3-times repeatedly and showed the best one. B: HASMCs were pretreated with various concentrations of THBA (0, 20, 50, and 100 μg/ml). Cells were incubated 2 h later with 100 ng/ml of TNF-α for 24 h. For the zymography assay, the media was subjected to gelatin SDS–PAGE. C: MMP-9 protein level decreased by THBA (0, 20, 50, 100, 200, 500 μg/ml). The total cellular proteins were prepared as per Materials and Methods Section and used in the Western blot analysis. The bars represent the mean ± SE of intensity ratios (MMP-9/GAPDH) at least three independent experiments. Results were statistically significant ($^{*}P < 0.05$ and $^{**}P < 0.01$ compared with TNF-α only stimulation by using Student's *t*-test).

THBA INHIBITS THE PRODUCTION OF MMP-9 IN TNF-α-STIMULATED HASMCs

TNF-α (100 ng/ml) has been known to induce MMP-9 in VSMC [Moon et al., 2004] as well as stimulate signaling events which regulate a variety of biological functions. Therefore we examined the effects of THBA on the TNF-α-stimulated HASMCs in cell culture systems. HASMCs were incubated with serum-free media to induce quiescence followed by the addition of THBA to the media 2 h before the treatment with TNF-α. After 24 h incubation, the cells were harvested and the remaining media were subjected to gelatin zymography. The zymographic assay revealed that TNF-α-stimulation caused a substantial increase in the production of MMP-9 and the THBA treatments showed decreased levels of MMP-2 and MMP-9 (Fig. 2B). As shown in Figure 2A,B, the gelatinolytic activity of MMP-2 and MMP-9 was inhibited by THBA in a dose-dependent manner, and we further investigated the cellular production of MMP-9 in the THBA and TNF-α treated HASMCs by Western blotting. Interestingly, the production level of the MMP-9 decreased

with the treatment of THBA (Fig. 2C), suggesting that THBA has an inhibitory effect on the MMP-9 production of HASMC.

THBA INHIBITS MIGRATIONS OF TNF-α-STIMULATED HASMCs

MMP-9 plays significant roles in the degradation of extracellular matrix and proliferation of VSMCs in atherosclerotic pathogenesis [Cho and Reidy, 2002; Galis et al., 2002]. Because the up-regulation of MMP-9 expression was expected to contribute to an invasive cellular phenotype, we examined the invasiveness of HASMC upon treatment with various concentrations of THBA in the presence or absence of TNF-α as indicated in Figure 3. The migrated cells of TNF-α-induced HASMCs increased to 5.4× the levels of TNF-α-untreated HASMCs (Fig. 3A). Moreover the migrated cells of the HASMCs treated with THBA (100 μg/ml) decreased to levels similar to TNF-α-untreated or (−)-epigallocatechin-3-gallate (EGCG)-treated HASMCs in a dose-dependent manner (Fig. 3B). As evidenced by a Matrigel-plugged invasion assay, THBA blocked the invasiveness of TNF-α-induced HASMCs, suggesting that THBA blocks the invasiveness of TNF-α-induced HASMCs.

EFFECT OF THBA ON THE TRANSCRIPTIONAL ACTIVITY OF MMP-9 PROMOTER IN TNF-α-STIMULATED HASMCs

To confirm our findings, which suggest that THBA inhibits TNF-α-stimulated MMP-9 expression in HASMCs as measured by Western-blot analysis (Fig. 2C), we investigated the effect of THBA on the MMP-9 promoter activity and level of MMP-9 mRNA. To reveal the effects of THBA on the transcriptional level of MMP-9 mRNA, we performed RT-PCR analysis. The intensity of the amplified DNA fragment decreased in a dose-dependent manner (Fig. 4A). Because the activities of MMP-9 are also tightly regulated by endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMPs) [Stetler-Stevenson et al., 1996], the levels of expression of TIMP-1 and -2 RNA were assessed by RT-PCR. THBA had no effect on TIMP-1 and -2 mRNA expression at the concentrations tested (Fig. 4A).

To evaluate the effects of THBA on the MMP-9 promoter, we performed a transient transfection with the pGL2-WT-MMP9pro and analyzed the luciferase activities. The plasmid constructed with wild-type MMP-9 promoter (pGL2-WT-MMP9pro) was used in this study [Suh et al., 2006]. As shown in Figure 4B, the luciferase activity of TNF-α-stimulated transfectants increased to 8× the level of unstimulated transfectants. On the other hand, the luciferase activity of the transfectants treated with THBA (100 μg/ml) in the presence of TNF-α, were reduced to the basal levels compared to the TNF-α-stimulated or unstimulated transfectants. These results confirmed the inhibition property of THBA on the gene expression of MMP-9 in TNF-α-stimulated HASMCs.

THBA INHIBITS MAPK PHOSPHORYLATION IN A TIME-DEPENDENT MANNER

In the previous reports in our laboratory as well as others, the MAPK pathways were found to be important for MMP-9 expression in TNF-α-treated VSMCs [Goetze et al., 1999; Cho et al., 2000; Moon et al., 2004]. To examine the inhibition effect of THBA on the TNF-α-induced activation of MAPKs, we treated cells with THBA (100 μg/ml) at various time-intervals, and induced them with TNF-α for 10 min. The short pre-incubation times

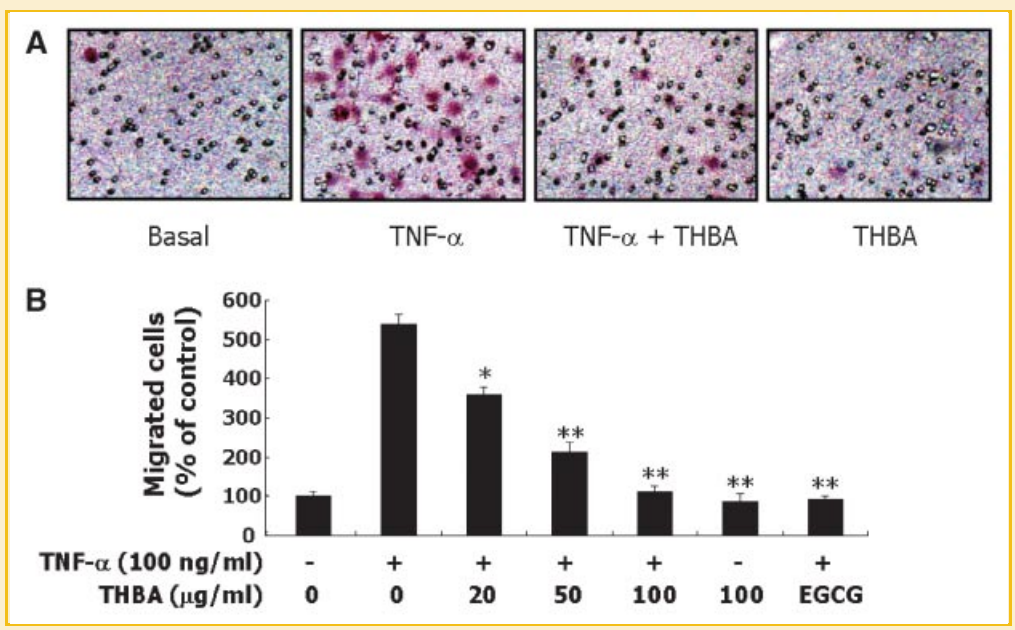

Fig. 3. THBA inhibits the migration of TNF-α-induced HASMCs. A: In the matrigel invasion assay, 500 μl of serum-free media containing TNF-α (100 ng/ml) was added to each well and Transwells with a matrigel coated membrane were inserted. Next, 200 μl of serum-free media containing HASMCs (2.5×10^5/ml) with various concentrations of THBA (0, 20, 50, 100 μg/ml) in the presence or absence of TNF-α (100 ng/ml) were placed in the upside of Transwell. After 24 h of incubation, the downward of the membrane were stained with hematoxylin and eosin Y. B: The stained cell numbers were counted as an average of three independent experiments. As a control, 80 μg/ml of epigallocatechin-3-gallate (EGCG) was used. Results are expressed as the mean ± SE of at least three independent experiments ($^*P < 0.05$ and $^{**}P < 0.01$ compared with TNF-α stimulation using Student's *t*-test). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

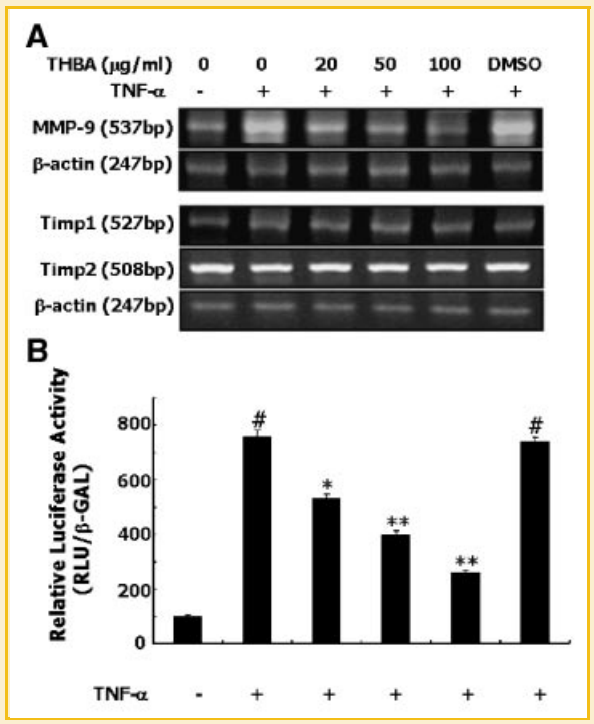

Fig. 4. THBA inhibits TNF-α-induced transcription and promoter activity of MMP-9, but do not inhibit the transcription of Timp-1 and Timp-2. A: RT-PCR was performed using MMP-9, Timp-1 and Timp-2 specific primers listed in Materials and Methods Section. HASMCs were treated with various concentrations of THBA (0, 20, 50, 100 μg/ml) in the presence or absence of TNF-α as indicated. B: Luciferase activity was measured in transiently transfected HASMCs using pWT-MMP-9pro as described in Materials and Methods Section. The transfected cells were treated with THBA (0, 20, 50, 100 μg/ml) in the presence or absence of TNF-α (100 ng/ml). Results are expressed as the mean ± SE of three independent experiments ($^{\#}P < 0.05$ compared with control, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with TNF-α treatment respectively, by using Student's *t*-test). In A and B, the 10 μl/ml DMSO was used as an inert control.

(from 30 s to 4 h) were enough to decrease the phosphorylation of ERK1/2, p38 and JNK MAPKs to the near basal levels in a time-dependent manner (Fig. 5A). Next, we tried to verify the p-ERK 1/2, p-p38 and p-JNK inhibition profile with time-courses (5, 15, 30 min) of TNF-α-stimulation after 2 h pre-incubation with THBA. The total cell lysates obtained from the HASMCs, which were treated with TNF-α for 5 and 15 min, showed increased levels of p-ERK1/2, p-p38 and p-JNK. Moreover, THBA remarkably suppressed the phosphorylation of these proteins (Fig. 5B) and the inhibition properties of THBA on the phosphorylation of ERK1/2, p-38 and JNK MAPKs led us to postulate that mechanisms regulating the inhibition of THBA on the migration and MMP-9 expression via MAPKs in HASMCs.

MAPKs PLAY CRUCIAL ROLES IN THE MMP-9 PRODUCTION AND INVASION IN HASMCs

To reveal the direct involvement of ERK1/2, p38 and JNK in the MMP-9 production, we used cell signal inhibitors such as U0126 (p-ERK inhibitor), SB203580 (p-p38 inhibitor), SP600125 (p-JNK inhibitor) and Wortmannin (PI3K inhibitor). We found that U0126, SB203580 and SP600125 inhibited the MMP-9 production in TNF-α-induced HASMCs, but Wortmannin did not (Fig. 5C). Because SP600125 was suspected to have non-specific inhibitory action to other kinases [Bain et al., 2003], we used another JNK inhibitor, I-JIP [Barr et al., 2002]. As demonstrated by Figure 5D, I-JIP failed to inhibit TNF-α-induced MMP-9 production sufficiently. However as revealed in Figure 5E, I-JIP inhibited the TNF-α-induced invasion. These results let us to postulate JNK might have important role in the migration of HAMSCs although it did not affect the MMP-9 expression. Furthermore to reveal direct involvement of MMP-9 on the HAMSCs invasion, we performed invasion assays using recombinant Timp-1 as specific MMP-9 inhibitor.

As shown in Figure 5E, inhibitors of MAPKs and Timp-1 have suppressive effect on the TNF-α-induced invasiveness of HASMCs. Because THBA is a potent inhibitory compound of p-ERK1/2, p-p38 and p-JNK MAPKs, the results led us to the conclusion that THBA is directly involved in MMP-9 production with these signal inhibitory action.

THBA INHIBITS NF-κB AND AP-1 NUCLEAR TRANSLOCATION INDUCED BY TNF-α

It has been known that MMP-9 gene expression in HASMCs is regulated by transcription factors such as NF-κB and AP-1. TNF-α is a potent activator of NF-κB and AP-1 and the putative binding sites of these transcription factors were located in MMP-9 promoter region [Moon et al., 2004]. To confirm whether THBA was directly involved with NF-κB and AP-1 mediated MMP-9 expression, we examined the binding of AP-1 and NF-κB isolated from TNF-α-stimulated HASMCs to oligonucleotides which contain specific sequences for the AP-1 or NF-κB binding sites using EMSA. The results of the EMSA showed that THBA suppresses the retarded electromobility shift complex from NF-κB and AP-1 induced by TNF-α (Fig. 6A).

Because NF-κB p65 and c-Jun are the main components of NF-κB and AP-1 complexes, we next examined the nuclear proportion of these transcription factors. As shown in Figure 6B, the Western-blot experiments revealed decreased levels of p65 and p-c-Jun proportions in the nuclear extract from HASMCs as a results of treatment with various concentrations of THBA (0, 20, 50, 100 μg/ml) in the presence or absence of TNF-α. In addition, we assessed phosphorylation levels of p65 and c-Jun in the cytoplasm in the condition of THBA treatment. HASMCs were treated with THBA (100 μg/ml) with different time-intervals as shown in Figure 6C and further incubated with TNF-α for 30 min. Western blot analysis using whole cell extracts indicated that THBA inhibited phosphorylation of p-p65 and p-c-Jun transcription factors in a time-dependent manner (Fig. 6C).

From our previous results indicating that MAPKs operated as upstream regulators of NF-κB and AP-1 in VSMCs [Moon et al., 2004], it is suggested that THBA modulate the NF-κB and AP-1 translocations via MAPK activity and have a direct involvement in the decreased performance of MMP-9 transcription.

DISCUSSION

As VSMCs are entirely surrounded by basal lamina and extracellular matrices in the media, VSMC migration requires the breakdown

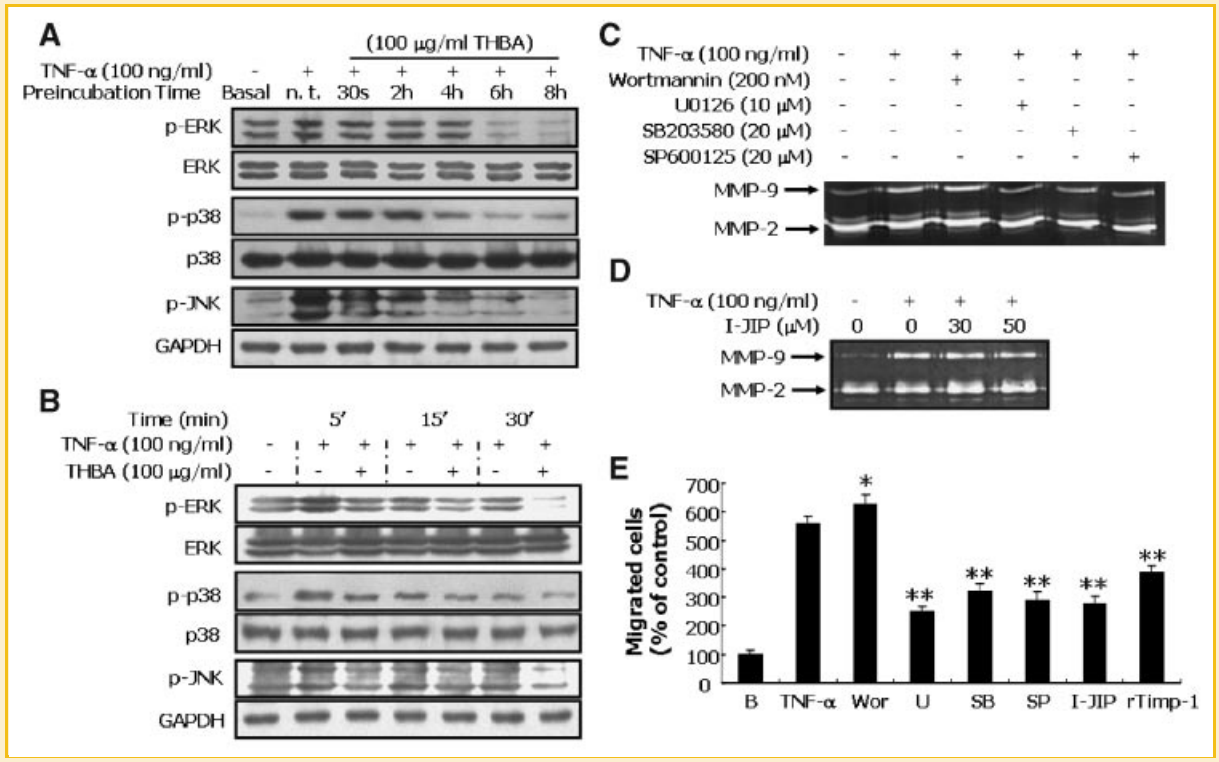

Fig. 5. THBA inhibits the phosphorylations of MAPKs. The inhibitors such as U0126, SB203580 and SP600125 inhibit MMP-9 expression and invasion of TNF-α-induced HASMCs. Another p-JNK inhibitor, I-JIP does not inhibit MMP-9 expression but it can inhibit invasion of HASMCs. A: THBA suppresses TNF-α-induced p-ERK1/2, p-p38 and p-JNK in a time-dependent manner. HASMCs were pre-incubated with THBA (100 μg/ml) for the indicated time period, and subsequently treated them with TNF-α (100 ng/ml) for 10 min at 37°C. The same amount of total cellular proteins were blotted to PVDF membranes and used for this experiment. ERK1/2, p38 and GAPDH were used as loading controls. n.t.; not treated. s: seconds, h: hours. B: In the time course analysis of TNF-α-induced HASMCs, THBA inhibits the phosphorylation of three MAPKs. HASMCs were pre-incubated with 100 μg/ml of THBA for 2 h, and subsequently treated with TNF-α (100 ng/ml) for 5, 15, and 30 min. Total cellular proteins were extracted and subjected to Western blot analysis with indicated antibodies. C: HASMCs were treated with cell signal inhibitors including Wortmnannin (200 nM), U0126 (10 μM), SB203580 (20 μM) and SP600125 (20 μM) and stimulated with TNF-α (100 ng/ml) 30 min later. After 24 h, the supernatants were subjected to gelatin SDS-PAGE. D: HASMCs were treated with various concentrations of I-JIP, a peptide inhibitor of p-JNK (H-GRKKRRQRRRPPRPKRPTTLNLFPQVPRSQDT-NH2) and stimulated with TNF-α (100 ng/ml) 2 h later. Gelatin SDS-PAGE was performed as described in Materials and Methods Section. E: Matrigel-plugged invasion assay was performed with 200 nM of Wortmannin (Wor), 10 μM of U0126 (U), 20μM of SB203580 (SB), 20 μM of SP600125 (SP), 50 μM of I-JIP and 100 nM rTimp-1 (recombinant Timp-1) as per Materials and Methods Section. Results are expressed as the mean ± SE of three independent experiments ($^{*}P < 0.05$ and $^{**}P < 0.01$ compared with TNF-α treatment using Student's *t*-test).

of the ECM [Dollery et al., 1995; Mason et al., 1999]. Among the extracellular proteinases, MMPs have been shown to play an essential role [Pauly et al., 1994; Dollery et al., 1995; Cho and Reidy, 2002]. Moreover the proliferation and migration of VSMCs play an important role in the pathogenesis of atherosclerosis and restenosis of the injured region. On injury, VSMCs migrate from the tunica media into the intima, leading to neointima formation [Ross, 1986, 1995]. With respect to cancer metastasis, MMP-9 activity also plays crucial roles by enhancing the action of cell motility, and hence the regulation mechanisms as well as the development of inhibitors for MMP-9 have been studied in atherosclerosis and cancers [Huang et al., 1994; Heber, 2001; Yao et al., 2001; Maeda et al., 2003; Chung et al., 2004].

In this article, we have investigated the inhibition property of THBA on the migration and the MMP-2 and -9 activities of HASMCs. We treated the supernatants of TNF-α-treated HASMCs with THBA in vitro and found the inhibition of MMP-9 and MMP-2 activities occurred in a dose-dependent manner. In parallel, as shown in Figure 2B,C, the TNF-α-induced MMP-9 production was inhibited by THBA. The activity of MMP-2 in the TNF-α-induced HASMCs remained unchanging as compared with the TNF-α untreated cells. Therefore, it is suggested that the decrease of MMP-2 activity in THBA treated HASMCs occurred as a result of direct inhibition of activity (Fig. 2B). To confirm whether THBA has an inhibitory influence on the MMP-9 gene expression, we performed RT-PCR analysis and promoter assay using 710 bp sequences of the 5'-flanking region of the MMP-9 gene with tagged luciferase activity. As shown in Figure 4A, the RT-PCR analysis indicated that THBA inhibited the expression of the MMP-9 gene and this result was confirmed by analyzing the lucifierase activity of the transfected pGL2-WT-MMP-9pro plasmid (Fig. 4B). These results highlight the dual inhibition mechanisms by the direct enzymatic inhibition of MMP-2, -9 activities and transcriptional inhibition of MMP-9 gene expression. TIMP-1 was known as a major inhibitor of MMP-9, but TIMP-1 and -2 are differentially regulated in vivo as well as cell in a culture systems [De Clerck et al., 1994]. Because Timp-1 and -2 mRNA were not changed by THBA (Fig. 4A), we ruled out the effects of THBA on TIMP-1 and -2.

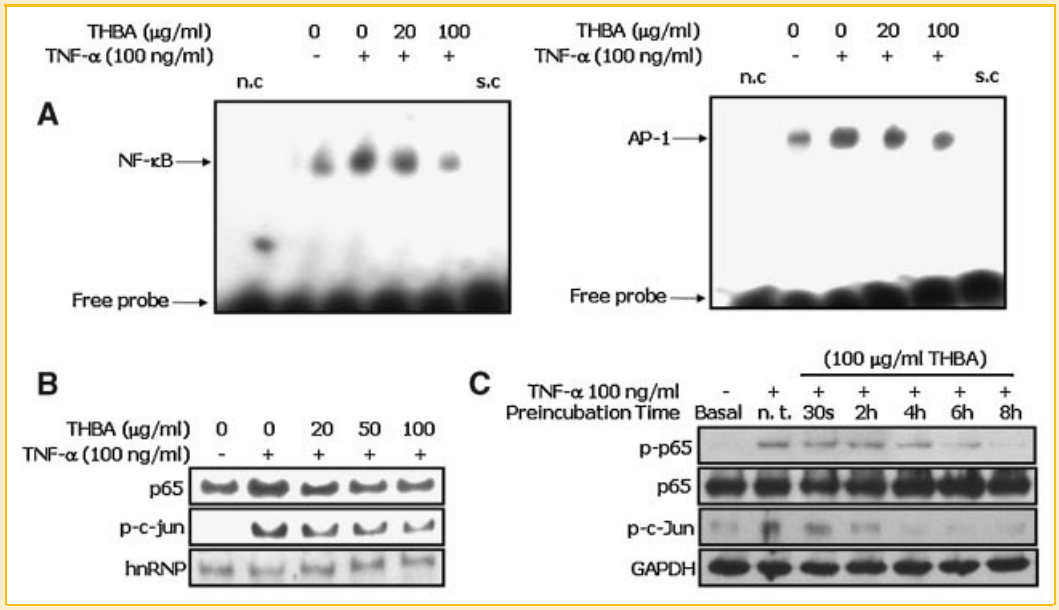

Fig. 6. THBA blocks the TNF-α-induced NF-κB and AP-1 nuclear translocations in a dose-dependent manner. A: The EMSA was performed with NF-κB and AP-1 specific probes. HASMCs were incubated with various doses of THBA (0, 20, 100 μg/ml) for 2 h, and then stimulated with TNF-α (100 ng/ml) as indicated. After 4 h, the nuclear extracts were prepared and subjected to the EMSA experiment as per Materials and Methods Section. (n.c; negative control; s.c, specific competeter, 100 fold of non-labelled specific probes were used as s.c). B: HASMCs were treated with various concentrations of THBA (0, 20, 50, 100 μg/ml) for 2 h, and then incubated with TNF-α (100 ng/ml) for 4 h. The nuclear extracts were prepared as per Materials and Methods Section. Western blot analysis was performed using prepared nuclear extract and demonstrated that THBA treatment decrease the proportion of the NF-κB p65 and p-c-Jun. C: THBA inhibits the TNF-α-induced phosphorylation of NF-κB p65 and c-Jun transcription factors in a time-dependent manner. At the 80% confluence of HASMC cultures, the media were changed with serum-free. The cells were treated with THBA (100 μg/ml) for the indicated times, and then incubated with TNF-α (100 ng/ml) for 30 min. The total cellular proteins were prepared and subjected to Western blot analysis. n.t.; not treated.

As shown in Figures 3 and 5, the invasion was occurred in the TNF-α-induced HASMCs and it was hypothesized the migration was involved with MMP-9 expression. We confirmed that the TIMP-1 protein, a known MMP-9-specific inhibitor, and EGCG, a known MMP-9 inhibitor, block the Matrigel invasion of TNF-α-induced HASMCs. Because TIMP-1 was bind to latent MMP-9 before secretion [Roderfeld et al., 2007], it is proposed TNF-α-induced MMP-9 gene expression caused MMP-9/TIMP-1 imbalance. Some report indicated 92 kDa MMP-9 was cleaved into active form MMP-9 (82 kDa) and these complex process was occurred when latent MMP-9 (92 kDa) was released from TIMP-1 protein and activated by plasmin [Dzwonek et al., 2004]. Therefore it is also proposed the high ratio of MMP-9/TIMP-1 causes MMP-9 activation and enhances matrix remodeling.

In our laboratory as well as others, it was demonstrated that TNF-α-induced transcription of MMP-9 was mainly mediated by AP-1 and NF-κB in VSMCs [Bond et al., 1998; Moon et al., 2004]. Furthermore, past studies revealed that the ERK1/2, p38 and JNK MAPKs were directly involved with MMP-9 production and cell migration in VSMCs and cancer cell lines [Cho and Reidy, 2002; Zhan et al., 2003; Moon et al., 2004]. Cho et al. [2000] demonstrated that the MAPK inhibitors, U0126 and SB203580 inhibit MMP-9 expression in VSMCs. Furthermore, Moon et al. [2004] indicated that Ras/ERK1/2 pathway was directly involved in MMP-9 expression by the way of suppressing NF-κB and AP-1. An in vivo experiment performed by Zhan et al. [2003] reported the possibility of p38-mediated hyperplasia as well as ERK1/2, p38 and JNK mediated migration in PDGF-induced rat aortic SMCs. In Figure 6, we demonstrated that AP-1 and NF-κB decreased in the nucleus as a result of THBA treatment. To explain inhibition mechanisms of THBA on the TNF-α-induced MMP-9 production via nucleus NF-κB and AP-1, we measured the phosphorylation event of ERK1/2, p38 and JNK in TNF-α-induced HASMCs. Using Western blot analysis, we found that the phosphorylations of ERK1/2, p38 and JNK decreased in the TNF-α-induced HASMCs treated with 100 μg/ml of THBA (Fig. 5A,B).

To substantiate the direct involvement between MAPK and HASMCs-invasion, we used inhibitors such as Wortmannin, U0126, SB203580 and SP600125. The results revealed that the MMP-9 production and cell invasion decreased when HASMCs were treated with MAPK inhibitors including U0126, SB203580 and SP600125 (Fig. 5C,E). These results highlight the significant role of MAPKs in MMP-9 expression and HASMC invasion. In addition, it could explain how the THBA-inhibited MAPK activity resulted in the decrease of MMP-9 secretion involving cardiovascular disease [Goetze et al., 1999; Cho et al., 2000]. In Figure 5C, wortmannin did not inhibit the MMP-9 expression in our systems, however it was reported that the wortmannin could inhibit the MMP-9 expression in VEGF-stmulated VSMCs [Yao et al., 2004]. In other hands, another p-JNK inhibitor, I-JIP failed to inhibit MMP-9 production (Fig. 5D) but it could inhibit the invasion of HASMCs (Fig. 5E). Intriguingly these results suggested different roles of JNK pathways in the MMP-9 expression and cell migrations by which it is proposed the MMP-9-independent mechanisms of migration [Turner et al., 2007]. Turner et al. reported MMP-9 dependent or independent invasions of human cardiac myofibroblasts. Both of siRNA for

MMP-9 and Simvastatin for inducing atypical organization of actin cytoskeleton resulted in the decrease of migration of human cardiac myofibroblasts. Also recombinant human MMP-9 treated human cardiac myofibroblasts showed increased invasions. In the case of I-JIP treated HASMCs, the blocking of invasion would have possibility of another mechanism related to invasion, however it deserves further investigation.

MMP-9 expression plays a prominent role for the cancer metastasis and atherosclerosis, and the development of drugs that have an inhibitory effect on MMP-9 activity might be one of the important areas in anti-cancer and anti-atherosclerosis therapy. THBA has a dual inhibition property because of its direct inhibition on the activities of MMP-2 and -9 as well as its modulation effect of regulation on the MMP-9 gene. In summary, we suggest that THBA has a strong potency for future therapeutic drugs of atherosclerosis because of its strong inhibitory action to MMP-9, MMP-2 as well as invasions in the TNF-α-induced HASMCs.

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