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Tanshinone IIA From *Salvia miltiorrhiza* BUNGE Inhibits Human Aortic Smooth Muscle Cell Migration and MMP-9 Activity Through AKT Signaling Pathway

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Abstract Smooth muscle cell (SMC) migration plays an important role in normal angiogenesis and is relevant to disease-related vascular remodeling in conditions such as brain arteriovenous malformations, pulmonary hypertension, arteriosclerosis, and restenosis after angioplasty. In this present study, we showed that tanshinone IIA, the major lipid-soluble pharmacological constituent of *Salvia miltiorrhiza* BUNGE, inhibits human aortic smooth muscle cell (HASMC) migration and MMP-9 activity. Tanshinone IIA significantly inhibited IκBα phosphorylation and p65 nuclear translocation through inhibition of AKT phosphorylation. Tanshinone IIA inhibited TNF-α-induced ERK and c-jun phosphorylation, but not other MAPKs such as JNK and p38. Tanshinone IIA also inhibited NF-κB and AP-1 DNA-binding. Moreover, tanshinone IIA inhibited the migration of TNF-α-induced HASMCs. Our results provide evidence that tanshinone IIA has multiple effects in the inhibition of HASMC migration and may offer a therapeutic approach to block HASMC migration. J. Cell. Biochem. 104: 15–26, 2008.

Key words: tanshinone IIA; Salviae Miltiorrahizae (*Salvia miltiorrhiza* Bunge); matrix metalloproteinase-9; human aortic smooth muscle cells; tumor necrosis factor-α

It has been shown that development of atherosclerosis is characterized by vascular endothelial cell (EC) dysfunction, proliferation and migration of vascular smooth muscle cells (VSMCs), and increased extracellular matrix deposition [Ross, 1993]. VSMC migration is believed to play a major role in the pathogenesis of many vascular diseases, including progressive intimal thickening after coronary intervention [Schwartz, 1997]. VSMC in the media has low mitogenic activity. During the early stages of arterial wall injury or atherosclerosis, aortic SMC may undergo transition from a contractile to a synthetic phenotype and begin proliferating in response to various growth factors and cytokines, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and tumor necrosis factor-α (TNF-α), among others, causing intimal

Abbreviations used: HASMC, human aortic smooth muscle cell; MMP-9, matrix metalloproteinase-9; TNF-α, tumor necrosis factor-α; MAPK, mitogen-activated protein kinase; PI-3K, phosphatidylinositol 3-kinase; AKT, AKT8 virus oncogene cellular homolog; NF-κB, nuclear factor kappa B; IκBα, inhibitor of NF-κB; AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; JNK, jun N-terminal kinase.

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thickening of the arterial walls [Ross, 1993; Motwani and Topol, 1998]. However, in addition to growth factor stimulation, the replication and migration of VSMC may require the degradation or remodeling of extracellular matrix (ECM) surrounding the cells [Matrisian, 1990; Sasaguri et al., 1994]. VSMC synthesizes important components of the ECM, including collagens, elastin, and proteoglycans [Galis et al., 1994; Strauss et al., 1994]. An imbalance between the accumulation and degradation of ECM may be crucial in the development of intimal thickening that form after vascular wall interventions [Strauss et al., 1994].

Matrix metalloproteinases (MMPs) are important for smooth muscle cell (SMC) proliferation and migration into the intima [Newby and Zaltsman, 2000]. An increased proteolytic activity in the vessel wall mediates the degradation of the ECM surrounding VSMC in response to injury [Newby and Zaltsman, 2000], a necessary step for permitting medial VSMC to migrate into the intimal space. MMPs comprise three main groups: the interstitial collagenases (MMP-1), the type IV collagenases or gelatinases (MMP-2 and -9), and the stromelysins (MMP-3) [Woessner, 1991]. Among these MMPs, gelatinases degrade denatured interstitial collagens, native basement-membrane collagens; the expression of MMP-2 and -9 has been implicated in the progression of atherosclerotic lesions [Newby and Zaltsman, 2000]. However, as described above, atherogenic lesions form during several pathological processes, which involve the accumulation of inflammatory cells and the release of cytokines [Abedi and Zachary, 1995]. TNF-α is a cytokine secreted by VSMC in the neointima after balloon-injury as well as by macrophages in atherosclerotic lesions [Tipping and Hancock, 1993; Clausell et al., 1995; Jovinge et al., 1997]. Recent reports from an in vivo study concluded that MMP-9 is critical for the development of arterial lesions by regulating both VSMC migration and proliferation [Galis et al., 2002]. On the basis of reports from several different laboratories, it has been generally concluded that the basal levels of MMP-9 are usually low, and that its expression can be induced by treatment of VSMC with TNF-α [Galis et al., 1994; Moon et al., 2003b]. Recent our results demonstrated that ERK1/2 mediates TNF-α-induced MMP-9 expression in VSMC via the regulation of NF-κB and AP-1 [Moon et al., 2003b].

Several natural products have been used for vascular diseases and also employed for treatment of atherosclerosis [Heber, 2001; Yoshie et al., 2001; Kim et al., 2003]. *Salvia miltiorrhiza* Bunge, a member of sage plant, is a well-known traditional medicine used for the treatment of cardiovascular diseases in Asia. Recent studies have showed that *S. miltiorrhiza* BUNGE significantly inhibits intimal hyperplasia and attenuates restenosis [Chen et al., 2001]. Tanshinones are the hydrophobic pharmacological components, which have been isolated from *S. miltiorrhiza* BUNGE. Among tanshinones in *S. miltiorrhiza* BUNGE, cryptotanshinone, tanshinone I, and tanshinone IIA are present in the greatest amount [Shia et al., 2005]. Especially, tanshinone IIA is a derivative of phenanthrene-quinone, which is a traditional herbal medicine that is used to treat cardiovascular diseases [Chen et al., 2001]. Therefore, we carried out this study to elucidate the inhibitory potential of tanshinone IIA on human aortic smooth muscle cells (HASMCs) migration and MMP-9 production induced by TNF-α treatment [Yuan et al., 2003].

MATERIALS AND METHODS

Materials

Dried root of *S. miltiorrhiza* Bunge (Chinese name: Danshen) was purchased from Kyungdong pharmaceutical market in South Korea. The tanshinone IIA was dissolved in dimethyl sulfoxide (DMSO). Recombinant human TNF-α was obtained from R&D Systems (Boston, MA). The monoclonal antibodies and polyclonal antibodies to p-ERK1/2, p-SAPK/JNK, and p-p38 were purchased from New England Biolabs (Beverly, MA). [γ^{32}]P-ATP was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). NF-κB (p65), p-c-jun, p-IκB(, p-AKT, and AKT antibodies were purchased from Santa Cruz Biotechnology (CA).

Preparation of Tanshinone IIA From *Salvia Miltiorrhiza* BUNGE

The plant materials were pulverized and extracted with methanol (100 ml) thrice. Methanol soluble fractions were concentrated under vacuum and the obtained residue (1.25 g) was subjected to chromatography. Column chromatography was performed on a 5350-cm column packed with silica gel 60 (finer than 200 and 100–200 mesh, Merck). The column was packed

with benzene, and the sample was loaded in benzene, and then eluted with benzene, followed by consecutive elution with benzene containing ethyl acetate 2%, 5%, 8%, 10%. Fractions eluted with 8% ethyl acetate were concentrated. Further purification through column chromatography followed by preparative TLC (benzene/ethyl acetate; 8:2) resulted in an orange red solid (20 mg). Structure of the compound (Fig. 1A) was established based on NMR and mass spectral data and by comparison with those of authentic sample.

Cell Culture

HASMCs were our deposit [Moon et al., 2003b], which was purchased from Bio-Whittaker (CA). HASMCs were cultured in SMC growth medium-2 containing 10% FBS, 2 ng/ml human basic fibroblast growth factor, 0.5 ng/ml human EGF, 50 μg/ml gentamicin, 50 μg/ml amphotericin-B, and 5 μg/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. The secreted albumin was served as a loading control to normalize the amount of total secreted proteins.

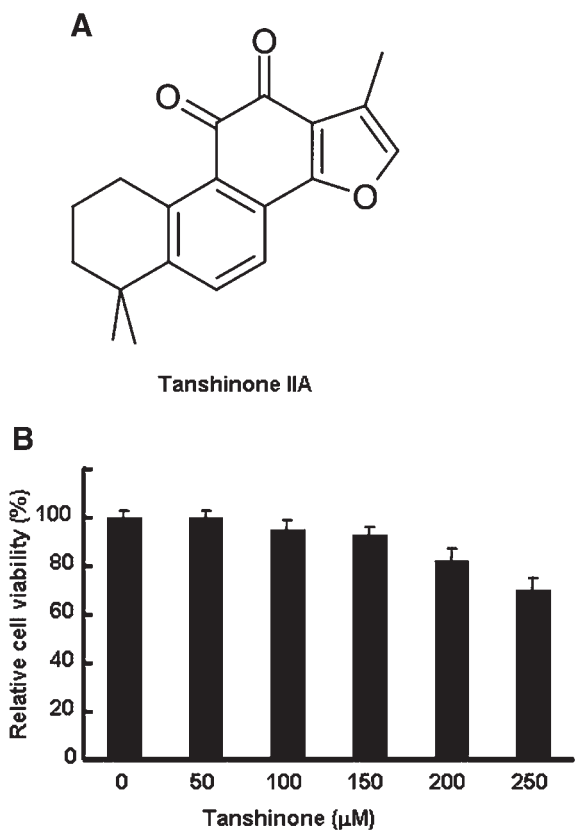

Fig. 1. **A**: The chemical structure of tanshinone IIA from *Salvia miltiorrhiza* BUNGE. **B**: Effect of tanshinone IIA on viability of HASMCs. In cell viability assay, after treatment of serum-starved HASMCs with various concentrations of tanshinone IIA (50, 100, 150, 200, and 250 μM) for 20 h in triplicates, the XTT solution was added and the optical density was read at 490 nm wavelength in a ELISA plate reader after 4 h incubation. The data were shown means ± SE as percent of control.

Electrophoretic Mobility Shift Assay (EMSA)

The nuclear extract of each cell 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 were 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 (5′-CTGAC CCCTGAGTCAGCACTT-3′), and NF-κB (5′-CCAGTGGAATTCCCCAG-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 AP-1, 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 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.

Cell Viability Assay

The cytotoxic effect of the tanshinone IIA 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 plates at a density of 1×10^4 cells/well in DMEM culture medium and allowed to attach for 24 h. After incubation the medium were discarded and replaced with 100 μl of new medium containing various concentrations of tanshinone IIA. After 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 in proportion 50:1) was added to the wells. The optical density was read at 490 nm wavelength in an ELISA plate reader after 4 h incubation of the plates with XTT in an incubator (37°C and 5% CO_2 + 95% air). All determinations were confirmed using replication in at least three identical experiments.

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 Becton–Dickinson (NJ). HASMCs (5×10^4 cells/well) to be tested for invasion were detached from the tissue culture plates, washed, resuspended in conditioned medium collected from without-TNF-α or TNF-α-treated or tanshinone IIA (0, 50, 100 μM) + TNF-α treated to HASMCs for 24 h, and then added to the upper compartment of the invasion chamber. Five hundred microliters of the same conditioned medium was added to the lower compartment of the invasion chamber. Cells without TNF-α-treated conditioned medium served as control. 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, and the cells on the upper side of the filter were removed using cotton swabs. The filters were fixed, mounted, and stained according to the manufacturer's instructions. The cells that invaded through the Matrigel and were located on the underside of the filter 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. 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 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 distilled pure water. For inhibitory effect of tanshinone IIA on TNF-α-induced MMP-9 expression, HASMCs were treated with various concentrations of tanshinone IIA in the presence of 100 ng/ml TNF-α and MMP-9 expression was evaluated by zymography. HASMCs were grown in 10% FBS/DMEM and rinsed with PBS, then incubated in serum-free DMEM with or without tanshinone IIA in the presence of TNF-α for 24 h and the conditioned media were collected. The conditioned medium was 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 proteolysis of the gelatin substrate. Bands corresponding to activity were visualized by negative staining using Coomassie Brilliant blue R-250 (Bio-Rad, Richmond, CA) and molecular weights were estimated by reference to prestained SDS–PAGE markers and MMP standards (Chemicon). Proteolysis was detected as a white zone in a dark field and the intensity of the bands obtained from zymogram studies was estimated with Scion Image (Scion, MA). The values are calculated by percent of control and expressed as means ± SE.

Statistical Analysis

Statistical analysis was performed using Student's *t*-test and $P < 0.01$ was considered

significant. All determinations were confirmed using replication in three independent experiments. The intensity of the bands obtained from autoradiography and Western blot was estimated with Scion Image (Scion). The values are calculated by percent of control and expressed as means ± SE.

RESULTS

Cytotoxicity of Tanshinone IIA on HASMCs

The cytotoxicity of tanshinone IIA on the HASMCs was evaluated using XTT cell proliferation assay kit. The HASMCs (5×10^4 cells/well) were incubated for 24 h in cultures in 96-well microplates with various final concentrations of tanshinone IIA (0, 50, 100, 150, 200, and 250 μM). Dose-dependent cytotoxic effect of tanshinone IIA against HASMC was shown in Figure 1B. We determined proper concentrations of tanshinone IIA for treatment. Hundred micromolars concentration of tanshinone IIA has a little cytotoxic effect on HASMC and used in this study.

Effect of Tanshinone IIA on the MMP-9 Activity

To examine the inhibitory activity of tanshinone IIA MMP-9 activity, the HASMCs were incubated with serum free media to induce quiescence and tanshinone IIA with various concentrations (0–100 μM) was added to the media in 1 or 2 h before treatment of TNF-α (100 ng/ml). After further incubation for 24 h, the media was harvested and introduced to zymography. As shown in Figure 2, both MMP-2 and MMP-9 were detected in the conditioned media of HASMCs, while TNF-α significantly increased the MMP-9 activity. Tanshinone IIA pretreatment inhibited MMP-9 activity in a concentration-dependent manner. At 100 μM, tanshinone IIA reduced MMP-9 activity to the basal level. In contrast, the activity of MMP-2 was not significantly affected by tanshinone IIA, or by TNF-α (Fig. 2). Additionally, when the media collected from TNF-α stimulated HASMC was treated with tanshinone IIA for 24 h, the MMP-9 activity was not affected (data not shown).

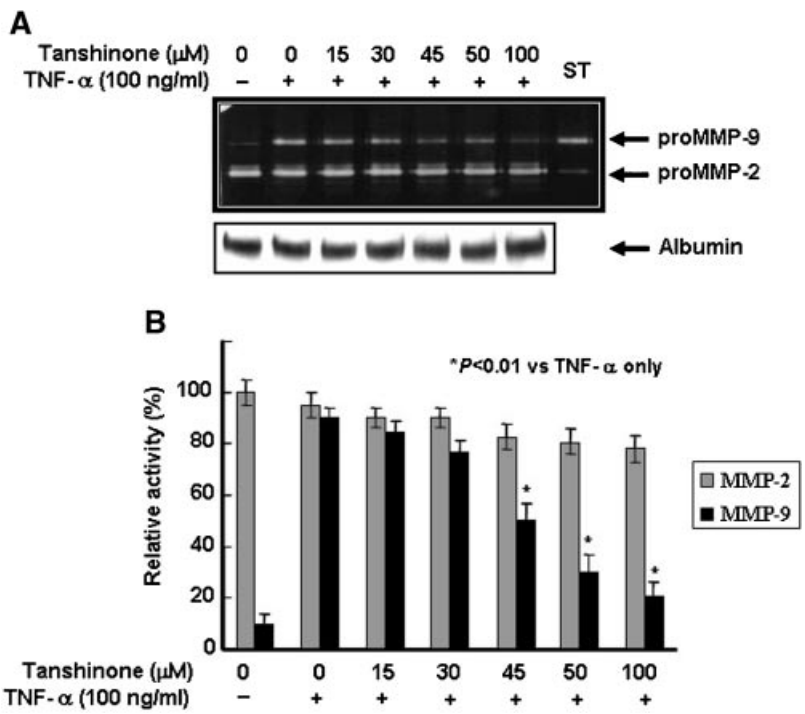

Fig. 2. Effect of tanshinone IIA on the MMP-2, and MMP-9 activity of TNF-α-induced HASMCs. **A**: Zymography was performed with conditioned media collected from HASMCs cultured in the presence or absence of TNF-α and tanshinone IIA (0, 15, 30, 45, 50, 100 μM). ST, MMP-2/9 marker (0.3 ng). The secreted albumin was served as a loading control to normalize the amount of total secreted proteins. **B**: The densitometric intensity of the zymography bands was estimated as described in Materials and Methods Section. The values are calculated by percent of control and expressed as means ± SE of three independent experiments.

Effect of Tanshinone IIA on AKT Phosphorylation and NF-κB Signaling

TNF-α activates AKT, which phosphorylates and activates IκB kinase (IKK), thus promoting NF-κB function [Ozes et al., 1999] and also, NF-κB is major transcription factor that regulates MMP-9 expression [Westermarck and Kahari, 1999]. We examined the effect of tanshinone IIA on AKT phosphorylation and NF-κB signaling. First, phosphorylated AKT was detected in TNF-α-induced HASMCs treated with or without 100 μM tanshinone IIA by various time courses. As shown in Figure 3A, phospho-AKT was increased by TNF-α treatment and tanshinone IIA strongly inhibited its increase.

It is well known that TNF-α activates NF-κB via IκB phosphorylation and degradation, followed by p65 nuclear translocation [Ghosh and Baltimore, 1990; Henkel et al., 1993]. We thus studied the effect of tanshinone IIA on the phosphorylation and degradation of IκB and nuclear translocation of p65 in TNF-α-stimulated HASMCs. As shown in Figure 3B, TNF-α treatment caused significant increase of phospho-IκBα. This induction was associated with an increasing translocation of p65 into the nucleus and decreasing cytosolic p65. Pretreatment of the cells with tanshinone IIA (0, 15, 30, 45, 50, 100 μM) for 2 h significantly inhibited both IκBα phosphorylation and p65 nuclear translocation in a concentration-dependent manner (Fig. 3B).

We examined the effect of tanshinone IIA on the DNA-binding activity of NF-κB. EMSA result revealed that TNF-α causes significant increase of NF-κB DNA-binding, and tanshinone IIA inhibits NF-κB DNA-binding in a concentration-dependent manner. The induced IκBα phosphorylation and p65 nuclear translocation are thus found to be consistent with the reduced NF-κB DNA-binding capacity (Fig. 3C).

Effect of Tanshinone IIA on c-jun Phosphorylation and AP-1 DNA Binding Activity

AP-1 is another major transcription factor that regulates MMP-9 expression [Westermarck and Kahari, 1999] and c-jun is one of the main components of transcription factor AP-1. It is well known that phosphorylated c-jun in nucleus is important for the binding and transcriptional activation of AP-1. Therefore, we determined whether c-jun phosphorylation could be inhibited by tanshinone IIA. The result indicates that TNF-α markedly enhance phosphorylated c-jun in nucleus, and tanshinone IIA significantly decrease phosphorylated c-jun in a concentration-dependent manner (Fig. 4A). To understand the possible mechanisms involved, we further examined the effect of tanshinone IIA on the DNA-binding activity of AP-1 using EMSA. TNF-α significantly increased DNA-binding activity of AP-1, and tanshinone IIA inhibited the binding activity in a concentration-dependent pattern (Fig. 4B).

Inhibitory Effect of Tanshinone IIA on the Phosphorylation of ERK, But Not JNK and p38

The MAPK pathways can influence AP-1 transactivation by increasing the level of AP-1 components or altering the phosphorylation of their subunits such as c-jun [Karin, 1995; Bode and Dong, 2000]. To determine which class of MAPK is involved in tanshinone IIA-mediated inhibition of AP-1 transactivation, we examined the effect of tanshinone IIA on the phosphorylation and activation of ERK, JNK, and p38 kinase. Our data showed that phosphorylation of MAPK occurred at 10 min after TNF-α treatment in HASMCs (Fig. 5). We thus investigated the effect of tanshinone IIA on JNK, ERK, and p38 phosphorylation in cells stimulated by 100 ng/ml TNF-α for 1 h. As shown in Figure 5, tanshinone IIA inhibited TNF-α-induced ERK phosphorylation at early time of induction (10 min), while having no evident effect on phosphorylation of JNK and p38. These results suggest that inhibition of ERK phosphorylation by tanshinone IIA is one of the underlying mechanisms involved in its inhibitory effect on AP-1 transactivation and the downregulation of MMP-9.

Suppressive Effect of Tanshinone IIA on TNF-α-Induced Migration of HASMCs

To determine whether tanshinone IIA could inhibit the migration of HASMCs, we used transwell plates to measure the cell migration following TNF-α stimulation. As shown in Figure 6A,B, tanshinone IIA inhibited the TNF-α-induced migration of HASMCs in a concentration-dependent manner.

DISCUSSION

In this present study, we showed that tanshinone IIA purified from *S. miltiorrhiza* BUNGE, at a nontoxic dose, inhibited HASMC

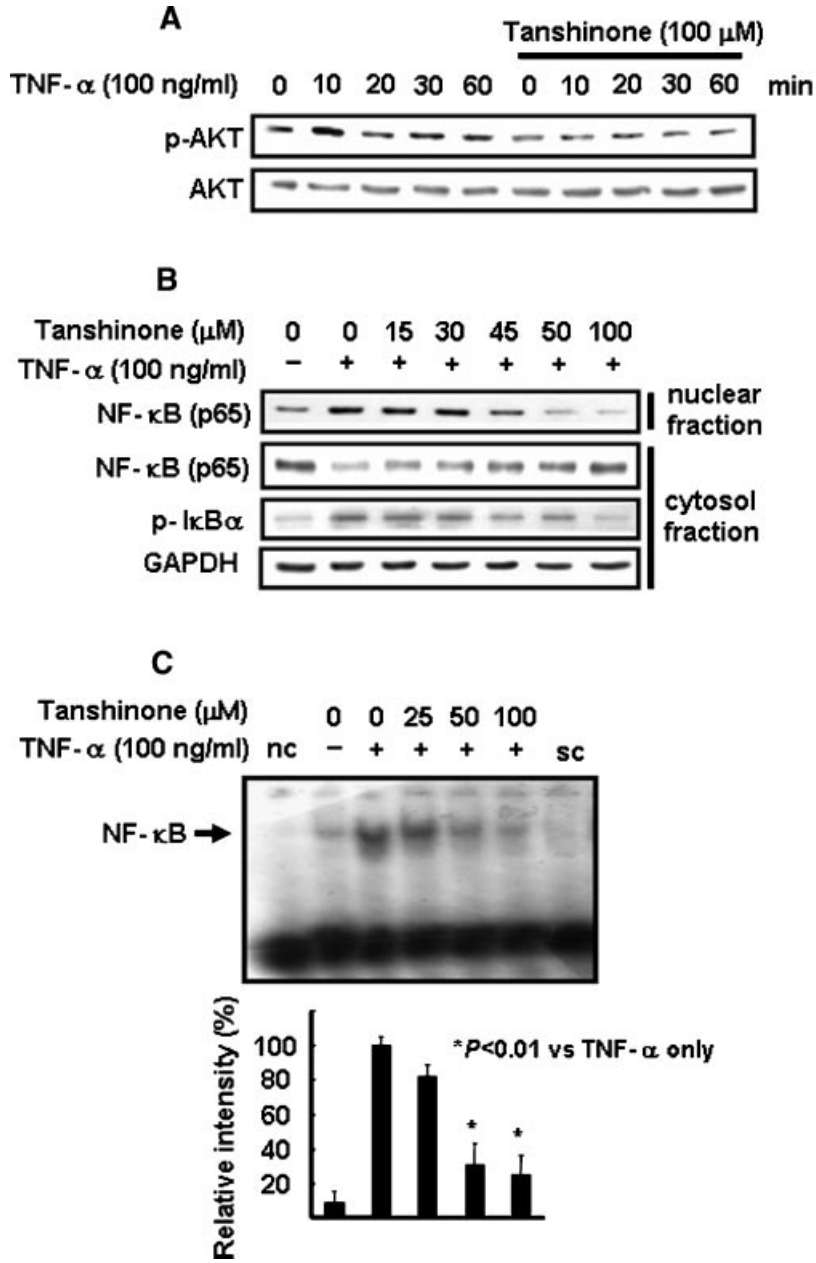

Fig. 3. Inhibitory effect of tanshinone IIA on NF-κB signaling pathway. **A**: Inhibitory effect of tanshinone IIA on TNF-α-induced AKT phosphorylation. Cells were pretreated with or without 100 μM tanshinone IIA for 2 h and then stimulated with or without 100 ng/ml of TNF-α for 1 h. The cytosolic extract was prepared and analyzed by Western blot. AKT (total AKT) is served as a loading control. **B**: Inhibitory effect of tanshinone IIA on TNF-α-induced p65 nuclear translocation and IκBα degradation. Cells were pretreated with 0, 15, 30, 45, 50, 100 μM tanshinone IIA for 2 h and then stimulated with or without 100 ng/ml TNF-α for 1 h. Cytosolic and nuclear proteins were prepared and analyzed by Western blot. GAPDH is served as a loading control. **C**: Concentration-dependent inhibition by tanshinone IIA on TNF-α-induced NF-κB DNA binding activity. The nuclear extract was subjected to EMSA as described in Materials and Methods Section. nc, negative control; sc, specific competitor for NF-κB. The densitometric intensity of the autoradiography bands was represented in bar graphs. The values are the means ± SE of three independent experiments.

migration and MMP-9 activity. Moreover, tanshinone IIA inhibited the activity of PI-3K/AKT and ERK1/2 signal pathway, but not other MAPK pathway. The concentration of tanshinone IIA necessary for the inhibition of these pathways was similar to those for the inhibition of HASMC migration. Tanshinone is the major lipid-soluble pharmacological constituent of

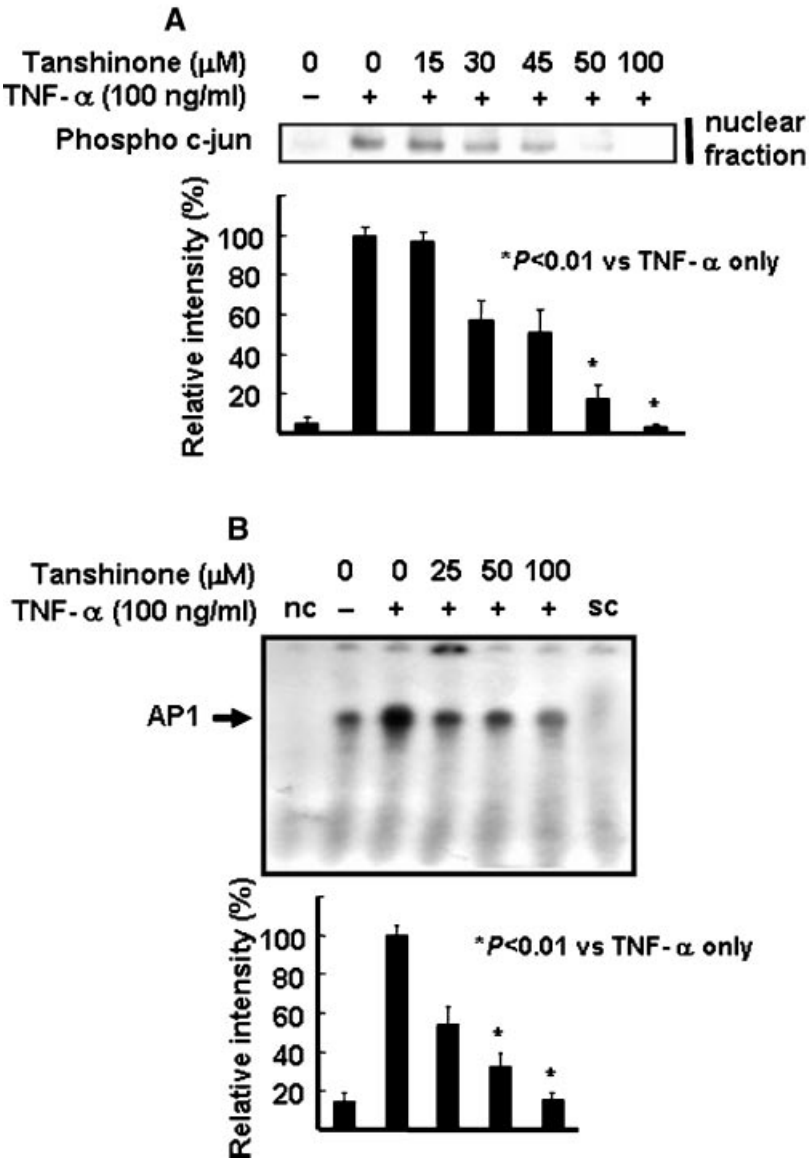

Fig. 4. Inhibitory effect of tanshinone IIA on AP-1 signaling pathway. **A**: Inhibitory effect of tanshinone IIA on TNF-α-induced c-jun phosphorylation in nucleus and (**B**) AP-1 DNA binding activity. Cells were pretreated with 0, 15, 30, 45, 50, 100 μM tanshinone IIA for 2 h and then stimulated with or without 100 ng/ml TNF-α for 1 h. Nuclear protein was prepared and analyzed by Western blot and EMSA as described in Materials and Methods Section. nc, negative control; sc, specific competitor for AP-1. The densitometric intensity of the Western blot and autoradiography bands was represented in bar graphs, respectively. The values are the means ± SE of three independent experiments.

S. miltiorrhiza BUNGE and tanshinone IIA is one of its main components. Previous studies demonstrated that tanshinone could efficiently prevent the neointima formation [Dua et al., 2005] and inhibits intima hyperplasia, which is mainly characterized with the proliferation and migration of SMC induced by abnormal hemodynamic changes [Li et al., 2004].

SMC migration plays an important role in normal angiogenesis and is relevant to disease-related vascular remodeling in conditions such as brain arteriovenous malformations, pulmonary hypertension, arteriosclerosis, and restenosis after angioplasty [Ross, 1995; Fanburg and Lee, 1997; Stenmark and Mecham, 1997]. SMC migration presumably requires degradation of the basement membrane and ECM surrounding the cell [Sankar et al., 1996]. As previous described, one family of such enzymes is MMPs which are zinc proteases that cleave

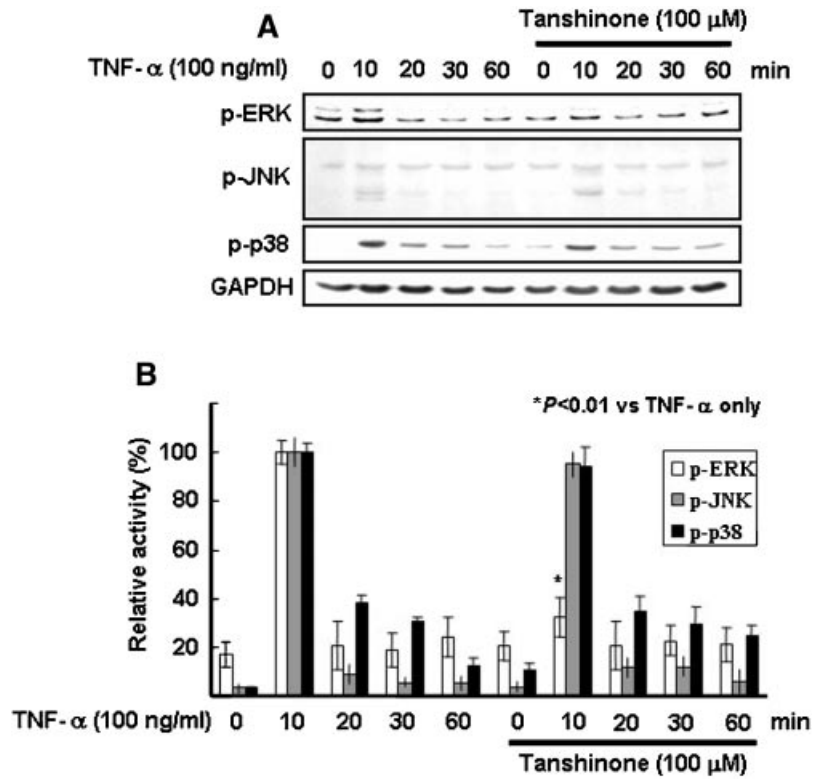

Fig. 5. Effect of tanshinone IIA on TNF-α-induced ERK, JNK, and p38 phosphorylation. **A**: Cells were pretreated with or without 100 μM tanshinone IIA for 2 h and then stimulated with or without 100 ng/ml TNF-α for 0, 10, 20, 30, and 60 min, respectively. The treated cells were harvested and analyzed by Western blot. GAPDH is served as a loading control. **B**: The densitometric intensity of the p-ERK, p-JNK, and p-p38 bands was normalized to GAPDH bands and represented in bar graphs, respectively. The values are the means ± SE of three independent experiments.

components of the ECM. Gelatinases such as MMP-2 and MMP-9 have been implicated in removing the first ECM barrier to migrating SMC [Pauly et al., 1994; Kenagy et al., 1997]. They have also been thought as a mediator of lesion development in response to vascular injury. MMP-9 was expressed within 6 h after carotid artery injury in rats and continues to be expressed for up to 6 days [Bendeck et al., 1994; Kenagy et al., 1997]. Although MMP-2 and MMP-9 have similar substrate specificities [Senior et al., 1991; Okada et al., 1992], regulation of their expression is different. For example, MMP-2 is constitutively expressed in SMC, and cytokines or growth factors could not induce its expression [Galis et al., 1994; Fabunmi et al., 1996]. In contrast, the basal levels of MMP-9 in SMC are usually low and cytokines or growth factors could induce its expression [Galis et al., 1994; Fabunmi et al., 1996]. Many studies identify increased expression of MMP-2 and MMP-9 coincident with SMC migration after vascular injury in vitro and in vivo [Bendeck et al., 1996]. It was demonstrated that MMP-2 could increase MMP-9 activity [Yong et al., 1998]. However, in our experiment, tanshinone IIA could weakly inhibit MMP-2 activity (Fig. 2), suggesting the downregulation of MMP-9 plays an important role in the inhibition of HASMC migration. We found that TNF-α can induce MMP-9 but not MMP-2 and tanshinone IIA can inhibit MMP-9 activity in TNF-α-treated HASMC.

The expression of MMP-9 is largely controlled at the transcriptional level [Westermarck and Kahari, 1999]. In the promoter region of MMP-9 gene there are two AP-1 binding sites and one NF-κB binding site [Sato and Seiki, 1993]. Mutations in AP-1 and NF-κB binding sites can reduce the induction of MMP-9 by phorbol ester [Chung et al., 2002; Moon et al., 2003c; Huang et al., 2004]. NF-κB is another nuclear transcription factor that is known to be important in the regulation of MMP-9 expression

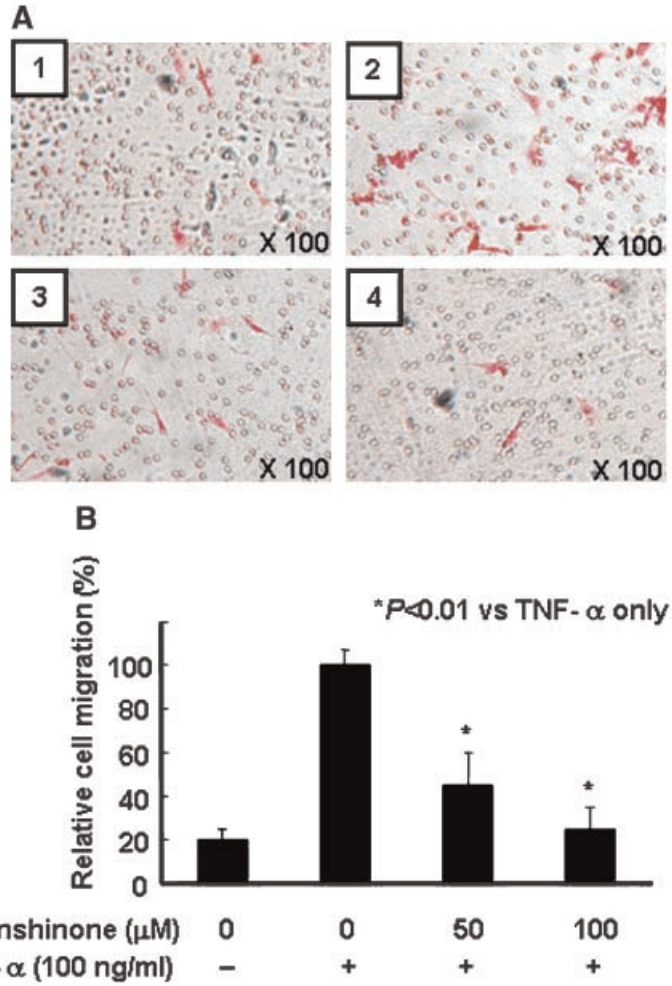

Fig. 6. Effect of tanshinone IIA on migration of TNF-α-induced HASMCs. HASMCs (5×10^4 cells/200 μl) and media treated with 100 ng/ml TNF-α and various concentrations of tanshinone IIA (0, 50, 100 μM) were added to Matrigel coated transwell. After 24 h incubation, underside of Matrigel filter was stained with hematoxylin and eosin Y. Cells were photographed at 100× using bright-field optics and the migrated cell number was counted as an average of three independent experiments. **A**: Microphotograph of migrated cells without TNF-α and tanshinone IIA (1), with only 100 ng/ml TNF-α (2), with 100 ng/ml TNF-α and 50 μM of tanshinone IIA (3), and with 100 ng/ml TNF-α and 100 μM of tanshinone IIA (4) were captured. **B**: The values were obtained and calculated by averaging the total number of migrated cells from three filters and expressed as means ± SE. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

[Sato and Seiki, 1993]. NF-κB is a transcription factor involved in multiple cellular processes, including cytokine gene expression, cellular adhesion, apoptosis, and metastasis [Bharti and Aggarwal, 2002]. In unstimulated cells, NF-κB is generally sequestered in the cytoplasm by the IκB proteins. When stimulated, IκB is phosphorylated and degraded. NF-κB, released from the inhibitors, translocates from cytoplasm into the nucleus, binds to the promoter region of genes containing its specific sites and activates gene transcription [Bharti and Aggarwal, 2002]. In this study, we demonstrated that tanshinone IIA suppressed TNF-α-mediated IκBα degradation and p65 nuclear translocation (Fig. 3B), thus inhibiting TNF-α-induced NF-κB DNA-binding (Fig. 3C).

On the other hand, it is well established that c-jun phosphorylation is mediated by MAPK pathways [Karin, 1995]. Generally, there are three classes of MAPKs: ERK, JNK, and p38 MAPKs. Activation of MAPK occurs through phosphorylation of specific threonine and tyrosine, and the components of AP-1, such as c-jun, can be phosphorylated after the translocation of activated MAPKs to the nucleus [Cowley et al., 1994]. Our data showed that tanshinone IIA inhibited the phosphorylation of ERK, but not JNK and p38 (Fig. 5). Since c-jun is the phosphorylation target of both JNK and ERK

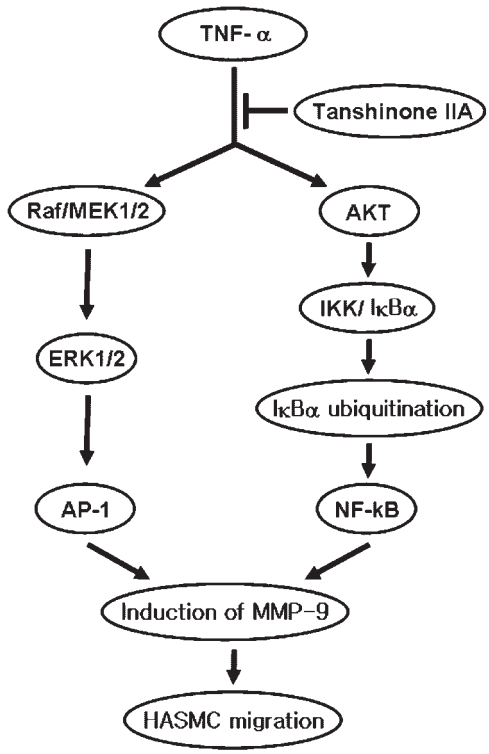

Fig. 7. Systematic action mechanism of tanshinone IIA on TNF-α-induced HASMC function.

[Bode and Dong, 2000], it is likely that tanshinone IIA inhibits the phosphorylation of c-jun through suppressing ERK activation but not JNK. At present, it is still not clear how tanshinone IIA affects MAPK signaling pathways. Several studies have demonstrated that TNF-α can activate the Raf/MEK1/2/ERK1/2 mitogenic cascade that plays an important role in regulating the MMP-9 expression [Moon et al., 2003a; Yao et al., 2004]. It is possible that this suppression leads to inhibition of TNF-α-induced ERK1/2 phosphorylation, followed by inhibition of MMP-9 expression and reduced invasiveness in TNF-α-treated HASMC.

Our present study demonstrates that tanshinone IIA directly inhibits TNF-α-induced HASMC migration and MMP-9 activities through downregulation of the PI3K/AKT and ERK1/2 signal pathway, as illustrated in Figure 7. Finally, the present evidence provide that tanshinone IIA has multiple effects in the inhibition of HASMC migration, suggesting downregulating MMP-9 production and MMP-9 enzyme activity, and inhibiting ERK1/2 and PI3K/AKt phosphorylation, may offer a therapeutic approach to block HASMC migration.

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