Tanshinone II A stabilizes vulnerable plaques by suppressing RAGE signaling and NF-κB activation in apolipoprotein-E-deficient mice

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Abstract. Tanshinone II A (TSIIA) is a diterpene quinone extracted from the roots of Salvia miltiorrhiza with anti-inflammatory and anti-oxidant properties that is used to treat atherosclerosis. In the current study, morphological analyses were conducted to evaluate the effects of TSIIA on atherosclerotic vulnerable plaque stability. Additionally, receptor of advanced glycation end products (RAGE), adhesion molecule, and matrix-metalloproteinases (MMPs) expression, and nuclear factor-κB (NF-κB) activation were examined in apolipoprotein E (apoE)-deficient mice treated with TSIIA. Eight-week-old apoE\textsuperscript{-} mice were administered TSIIA and fed an atherogenic diet for 8 weeks. TSIIA exhibited no effects on plaque size. Analysis of the vulnerable plaque composition demonstrated decreased numbers of macrophages and smooth muscle cells, and increased collagen content in apoE-deficient mice treated with TSIIA compared with untreated mice. Western blotting revealed that TSIIA downregulated the expression levels of vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and MMP-2, -3, and -9, suppressed RAGE, and inhibited NF-κB, JNK and p38 activation. The present study demonstrated that the underlying mechanism of TSIIA stabilization of vulnerable plaques involves interfering with RAGE and NF-κB activation, and downregulation of downstream inflammatory factors, including ICAM-1, VCAM-1, and MMP-2, -3 and -9 in apoE\textsuperscript{-} mice.

Introduction

Vulnerable atherosclerotic plaques are composed of a lipid-rich necrotic core covered by a thin fibrous cap predominantly comprised of smooth muscle cells (SMCs) and structural collagen, and such plaques are infiltrated with inflammatory cells (1). Inflammation has an important function in lesion destabilization and rupture, and macrophages are the major cellular components of vulnerable plaques, which are responsible for releasing numerous inflammatory cytokines, thus contributing to the progression of plaque vulnerability (2). Macrophages that infiltrate the plaques synthesize several proteolytic enzymes that are responsible for degrading lesion structure constituents. One such family of enzymes, matrix metalloproteinases (MMPs), efficiently degrade extracellular matrix (ECM) proteins (3). MMP-2, -3, and -9, in particular, degrade basement collagens, including elastin, laminin and gelatin, and have been implicated in the weakening of the fibrous cap (4,5). Macrophages can weaken advanced plaques by secreting proteases, primarily MMPs, which digest the ECM and collagen that provide the architectural structure and physical strength of the cap. Furthermore, studies have demonstrated that MMPs are distributed throughout the vulnerable regions and that their activities are significantly higher in unstable carotid plaques compared with the stable lesions (6-8). Thus, control of MMP activation is a potential therapeutic approach for targeting advanced plaques.

In the advanced stage of atherosclerosis, the receptor of advanced glycation end products (RAGE)-dependent signaling pathway is critical for chemokine and adhesion molecule generation, and also acts as an amplified step/amplifier for inflammatory processes (9). Studies have suggested that the ligation of RAGE and its ligands, including oxidized low-density lipoprotein and advanced glycation end products, may activate mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) to result in MMP upregulation, which accelerates erosion and thinning of the fibrous caps, and is responsible for plaque instability (10,11). Additionally, in macrophages, the important consequences of RAGE-dependent signaling include activation of NADPH-oxidase and MAPKs, which elevates the generation of reactive oxygen species.

Key words: MAPK signal pathway, tanshinone II A, atherosclerosis, matrix metalloproteinases, nuclear factor-κB, receptor of advanced glycation end products
(ROS), pro-inflammatory factors and RAGE itself, which further promotes this signal transduction, thus creating a positive feedback loop (12,13).

Furthermore, RAGE can induce the sustained activation of NF-κB, the fundamental transcriptional factor of the inflammatory response, by stimulating the degradation of NF-κB inhibitor α (IkBα) and synthesis of NF-κB p65, thus, ensuring a prolonged inflammatory response (14). Therefore, RAGE is considered to be a link between pro-inflammatory cytokine release and atherosclerosis initiation. Furthermore, oxidative stress leads to endothelial cell injury and vascular inflammation expansion by direct activation of the redox-sensitive transcription nuclear factor NF-κB via several mechanisms (15). Thus, the vicious circle between ROS and sustained activation of the RAGE axis perpetuates the damaging effects caused by chronic inflammation within the dysfunctional vascular wall. Therefore, suppression of ROS generation and RAGE expression may be a useful anti-atherosclerotic target.

Tanshinone II A (TSIIA) is the main lipoid soluble active ingredient of *Salvia miltiorrhiza*, a traditional Chinese herb, commonly used for the prevention and treatment of cardiovascular diseases, including atherosclerosis. Numerous studies in animal models and human patients have been demonstrated that TSIIA is an effective free-radical scavenger with anti-oxidant and anti-inflammatory properties, and is able to suppress the expression of adhesion molecules and chemokines. However, the precise mechanism of action of TSIIA on vulnerable atherosclerotic plaque stability has not been elucidated (16,17). The present study presents evidence that TSIIA inhibits the progression of advanced lesions via anti-oxidant/anti-inflammatory biological properties. TSIIA inhibits the expression RAGE and MAPK signaling pathway proteins, and NF-κB activation, leading to reduced expression of pro-inflammatory factors, including vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and MMPs in the advanced lesions of apolipoprotein E (apoE)−/− mice, which are the most used model mice for atherosclerosis due to their plasma cholesterol, triglycerides, chylomicron and very-low-density lipoprotein levels that are ~5-10 times than that of control mice. In addition, apoE−/− mice rapidly and easily develop the formation of advanced plaques (18,19).

**Materials and methods**

*Preparation of animals.* Eight-week-old male apoE−/− mice with a C57BL/6 J background were obtained from Peking University Health Science Center [purchased from Jackson Laboratory (Ben Harbor, ME, USA)]. The mice (n=16) were fed a high-fat, cholesterol-rich/atherogenic diet containing 21% fat, 19.5% casein, and 1.25% cholesterol for 8 weeks, and housed at 20-24°C and 45-55% humidity with a 12-h light-dark cycle. The mice were divided into a TSIIA group (n=8) and a control group (n=8). In the TSIIA group, TSIIA was dissolved in distilled water and administered daily by oral garage at a dose of 30 mg/kg for 8 weeks, and in the control group equal volumes of distilled water were used as described previously (17). TSIIA extracted from the roots of *Salvia miltiorrhiza* was purchased from Guangxi Wuzhou Pharmaceutical Group Co., Ltd. (Wuzhou, China). The chemical purity of TSIIA was ~97%. All mice were anesthetized by intraperitoneal injection with sodium pentobarbital (0.055 mg·kg−1) and dissected longitudinally, removing the heart, brachiocephalic arteries (BCA), descending arteries and blood. Blood samples were collected from the mice for the measurement of plasma glucose and lipid levels. All animal protocols were approved by the animal ethics committee of the China-Japan Friendship Hospital (Beijing, China).

*En face analysis of the descending aortas.* Six descending aortas of each group were subjected to en face lipid staining. The aortas were dissected from the left subclavian artery to the iliac bifurcation and subsequently opened longitudinally and stained with Oil Red O for 10 min to visualize the extent of the lipid deposition. Quantitative analysis of lesion size was performed by capturing images of the aorta using a digital camera (DXC-960MD; Sony Corporation, Tokyo, Japan), and then the data were analyzed using Image Pro Plus 6 software. Firstly, the bar was calibrated and the positive areas of immunohistochemical and Sirius Red stains were selected and counted precisely, then the data were outputted.

*Quantification of atherosclerotic lesions in the brachiocephalic artery.* The BCA were dissected and fixed overnight in 4% polymerized formaldehyde, embedded in paraffin, and sectioned to 5-μm thick as described previously (20). Every sixth section was stained with the modified Movat pentachrome stain (21) and Sirius Red (22). The atherosclerotic lesions were analyzed using Image Pro Plus 6 software (Media Cybernetics, Inc., Rockville, MD, USA).

*Immunohistochemical staining.* Every sixth section from the BCA and aortic root was subjected to immunohistochemical analysis to identify macrophages and SMCs, and to measure MAC-3 (CD107b) and α-actin expression. Briefly, the sections were blocked by normal goat serum for 30 min at room temperature and then incubated with polyclonal antibodies at 37°C for 60 min or at 4°C overnight and then with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) at 37°C for 60 min. Finally, the coverslips were mounted with 1,4-diazabicyclo[2.2.2]octane and analyzed using an upright fluorescent microscope (Carl Zeiss AG, Oberkochen, Germany). Antibodies against α-actin (sc-32763; 1:100 dilution) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA) and MAC-3, a macrophage marker (550292; 1:60 dilution), was purchased from BD Bioscience (Franklin Lakes, NJ, USA) and the HRP-conjugated antibody (PV6000) was purchased from Beijing OriGene Technologies, Inc. (Beijing, China). The bar was calibrated and the positive areas of immunohistochemical and Sirius Red stains were selected and counted precisely, then the data were outputted.

*Polarization microscopy.* Subsequent to staining with picrosirius red, the sections were imaged by using standard bright-field imaging in addition to polarized light microscopy according to the certified protocols. Firstly, a white light source and two polarizing filters were added (Carl Zeiss AG). The light passed through a polarizer, the sample and an analyzer. The birefringent material, particularly collagen, changes the polarization status of the light, resulting in increases or...
reductions of the light intensity observed, depending on the relative orientation through the sample and the filters (23).

Western blotting. The descending arteries were dissected and subjected to western blotting for protein level analysis. The arteries mixed with RIPA were ground in a homogenizer on ice to form homogeneous lysates, then were centrifuged at 14,000 x g for 10 min at 4°C. The supernatants were removed from the cellular debris and protein concentration was determined using the Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology, Inc., Nanjing, China) (24). The lysates (10-30 µg of protein) were separated by using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA), blocked with 5% nonfat dry milk for 60 min, and probed with antibodies at 4°C overnight. The blots were incubated with HRP-conjugated anti-IgG for 1 h at 37°C, followed by detection with electrochemiluminescence reagents (Santa Cruz Biotechnology, Inc.). Antibodies against IκBα (sc-847; 1:1,000 dilution), phospho-IκBα (sc-7977; 1:500 dilution), IκBα (sc-10736; 1:500 dilution), MMP-2 (sc-31074; 1:500 dilution), MMP-3 (sc-20517; 1:500 dilution), VCAM-1 (sc-8304; 1:1,000 dilution), β-actin (sc-130656; 1:1,000 dilution), galectin-3 (sc-20517; 1:3,000 dilution) and MCP-1 (sc-28879; 1:1,000 dilution) were purchased from AbD Serotec ( Raleigh, NC, USA) (#4668; Thr183/Tyr185; 1:1,000 dilution), JNK (#9252; 1:1,000 dilution), phospho-JNK (#4611; Thr180/Tyr182; 1:1,000 dilution), p38 (#8690; 1:1,000 dilution), phospho-p38 (sc-3776; 1:500 dilution), IκBα (sc-10736; 1:1,000 dilution), IκBα (sc-10736; 1:500 dilution), IκBα (sc-10736; 1:500 dilution), VCAM-1 (sc-8304; 1:1,000 dilution), β-actin (sc-130656; 1:1,000 dilution), galectin-3 (sc-20517; 1:3,000 dilution) and MCP-1 (sc-28879; 1:1,000 dilution) were purchased from Santa Cruz Biotechnology, Inc. The antibodies against RAGE (SABI401326; 1:500 dilution) and MMP-9 (AV33090; 1:1,000 dilution) were obtained from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Antibodies against phospho-JNK (#4668; Thr183/Tyr185; 1:1,000 dilution), JNK (#9252; 1:1,000 dilution), phospho-p38 (sc-4511; Thr180/Tyr182; 1:1,000 dilution), p38 (#8690; 1:1,000 dilution), phospho-ERK1/2 (#4370; Thr202/Tyr204; 1:1,000 dilution), ERK1/2 (#4695; 1:1,000 dilution), NF-κB p65 (#8242; 1:1,000 dilution), and phospho-NF-κB (#3033; Ser536; 1:1,000 dilution) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA), while antibody to CD68 (R&D Systems, MN, USA) was purchased from Santa Cruz Biotechnology, Inc. (Raleigh, NC, USA). Antibodies against MCP-1 (P<0.01) expression levels were also decreased by TSIIA treatment, as demonstrated by immunohistochemical staining. Furthermore, SMCs were predominantly parallel with the collagen and distributed in the fibrous cap (Fig. 2A). All of these factors facilitate lesion stability.

Statistical analysis. All values are represented as the mean ± standard error of the indicated number of measurements. Unpaired Student's t-tests and analysis of variance for repeated measures were conducted in SigmaPlot software, version 13.0 (Systat Software, Inc. San Jose, CA, USA), and P<0.05 was considered to indicate a statistically significant difference.

Results

TSIIA decreases MMP-2, -3 and -9 expression by suppressing RAGE and activating the MAPK signaling pathway. The present study also investigated the mechanisms underlying elevated collagen content in the plaques from BCA in apoE−/− mice administered with TSIIA. MMP-2, -3 and -9 are critical enzymes for collagen degradation. Western blotting revealed that TSIIA treatment reduced MMP-2, -3 and -9 expression in the descending arteries compared with control levels (P<0.05; 25,26). The results of the present study demonstrated that, compared with the model group, the phosphorylation of IκB (P<0.05) and NF-κB (P<0.05) were decreased, and the total level of IκB was increased (P<0.05) in the descending arteries of apoE−/− mice treated with TSIIA (Fig. 3C), indicating that the anti-inflammatory abilities of TSIIA, at least partially, depend on the inhibition of NF-κB activation.
Fig. 4A). These results indicate that TSIIA-elevated collagen content be caused by downregulation of MMPs in the vulnerable plaques. Furthermore, previous studies have indicated that RAGE and the MAPK signaling pathway are responsible for NF-κB activation and MMP expression. The expression levels of MMPs, RAGE and phospho-MAPK protein in the descending arteries were determined using western blotting. Phosphorylation activates JNK, ERK1/2 and p38, and western blotting revealed that TSIIA administration reduced, but did not abolish, the phosphorylation of JNK, ERK1/2 and p38 compared with control levels (Fig. 4B). Furthermore, the expression levels of RAGE were assayed using western blotting, and the results demonstrated that TSIIA administration reduced the RAGE expression levels compared with control levels (P<0.01; Fig. 4C). Taken together, these results suggest that TSIIA decreased MMP and RAGE expression and suppressed MAPK signaling pathway activation, which may have resulted in vulnerable plaque stabilization.

Discussion

The present study presented experimental evidence that TSIIA stabilizes vulnerable atherosclerotic lesions in apoE-/- mice. In particular, the results demonstrated: i) The inhibitory effects of TSIIA on MMP expression and activation; ii) the association between TSIIA-induced suppression of NF-κB, VCAM-1, ICAM-1 and MCP-1, and reduced atherosclerotic plaque inflammation; and iii) the TSIIA-induced suppression of RAGE and downstream MAPK signaling pathways.

Morphological analysis has indicated that vulnerable plaque stability is more heavily dependent on plaque composition than the degree of stenosis. Unstable lesions are characterized by a distinct lipid necrotic core under the weakened fibrous cap, which protects the necrotic core from physical stress and inflammatory cell invasion (1,2). ECM components, collagen and elastin in particular, constitute an architecture that maintains fibrous cap integrity; thus, the balance between the synthesis and degradation of these matrix components is responsible for plaque vulnerability (27). Macrophages provide the major inflammatory factor source in vulnerable lesions and are thought to control this balance due to their secretion of enzymes that digest the ECM, particularly the MMP family (28).

How do macrophages contribute to remodeling unstable lesions? A series of studies revealed the tight correlation between macrophage infiltration in rupture-prone atheroma, such as the vulnerable shoulder areas, and the thinning of the fibrous cap and local MMP activation and accumulation, particularly MMP-2, 3 and 9 and collagenases that degrade collagen (5,29). The MMP-induced digestion of collagen and other ECM components weakens the protective caps and provides a path for the migration of inflamed cells from the circulation, and furthermore, facilitates the release of matrix-bound bioactive molecules, including fibroblast growth factor-2 and transforming growth factor-β (30). Therefore, suppressing MMP activity may be an effective treatment for atherosclerosis. In human and animal models of atherosclerosis, levels of various MMPs, including MMP-2, -3, -7, -9 and -12, are increased in advanced plaques. In fact, abundant MMPs have been identified in vulnerable regions of human plaques (28,31). Evidence from human epidemiological and genetic experiments revealed that MMP-9 is most closely associated with plaque instability and clinical manifestations. Only activated macrophage-secreted MMP-9 is sufficient to induce the rupture of unstable lesions atherosclerotic mice, and MMP-9-induced proteolysis is central to the rupture of human plaques (4,30).
Figure 2. TSIIA decreases the counts of macrophages, major harmful cellular sources in advanced lesions, within the BCA lesions. (A) Immunohistochemical staining for MAC-3 (CD107b) and α-actin, picrosirius staining and polarization microscopy were performed on BCA samples from the model and TSIIA group. The amounts of (B) macrophages, (C) SMC and (D) collagen in the lesions within the BCA were determined by immunohistochemical and picrosirius staining (n=6; P=0.0327, 0.0934 and 0.0111). Data represent the mean ± standard error. *P<0.05, **P<0.01 vs. model group. BCA, brachiocephalic artery; TSIIA, Tanshinone II A; SMC, smooth muscle cell.

Figure 3. TSIIA suppresses NF-κB activation and downregulates inflammatory factors, adhesion molecules and MCP-1. (A) Expression levels of macrophage markers, galectin-3 and CD68, in descending arteries of apoE−/− mice was detected by western blotting (P=0.0157 and P=0.0274). (B) Expression levels of VCAM-1, ICAM-1 and MCP-1 in the descending arteries were detected using western blotting (P=0.0218, 0.0223 and 0.0018). (C) NF-κB activation in the descending arteries was analyzed by measuring NF-κB, p-NF-κB, IκBα, and p-IκBα levels (P=0.2743, 0.0234, 0.00428 and 0.034). Data represent the mean ± standard error (n=4). *P<0.05, **P<0.01 vs. con. Con, control; TSIIA, Tanshinone II A; VCAM-1, vascular cell adhesion molecule; ICAM-1, intercellular adhesion molecule; MCP-1, monocyte chemoattractant protein-1; p-, phosphorylated; NF-κB, nuclear factor-κB; IκBα, NF-κB inhibitor α.
Macrophages also secrete MMP-2, which degrades collagen within the fibrous caps of human advanced plaques (32,33). Silence et al (5) demonstrated that MMP-3 destabilizes plaques by degrading matrix components, and also enhances macrophage accumulation and migration in the lesions, potentially by secreting urokinase and plasmin, critical activators of macrophage-secreted pro-MMPs, and causes the digestion of elastin and collagen. Furthermore, MMP-3 has similar proteolytic abilities to those of MMP-9, but also may directly determine MMP-9 and MMP-2 activities. The current study demonstrated that MMP-2, -3 and -9 were reduced and collagen was increased in vulnerable lesions in apoE−/− mice administered with TSIIA, indicating that increased collagen content following TSIIA treatment is associated with resistance to degradation by proteolytic enzymes, particularly the MMPs, major enzymes of the ECM digestion process. However, TSIIA decreased MMP levels (Fig. 4A) were accompanied by reduced macrophage accumulation (Fig. 2B) using its inflammatory inhibition abilities. ECM is primarily synthesized by SMCs within the plaques. The present study demonstrated that the number of SMCs in the plaques was increased by TSIIA treatment, although the difference between the treatment and control groups was not significant (Fig. 2C). As SMCs are the only cellular source of collagen, TSIIA stabilizes the advanced plaques, at least partially, by increasing the number of SMCs within the plaques.

Adhesion molecules and chemokines, including ICAM-1, VCMA-1 and MCP-1, are important cytokines that determine macrophage infiltration and accumulation (34). The activation of the redox-sensitive transcription, NF-κB, leads to directly increased expression pro-inflammatory cytokines and adhesion molecules. In atherosclerotic conditions, NF-κB is a well-known transcription factor with an important role in regulating the expression of inflammatory factors, including cytokines, chemokines, adhesion molecules and MMPs, in macrophages (35). In resting cells, the NF-κB dimer is located in the cytoplasm, bound to its inhibitory protein, IκB. Activation of IκB kinase, which induces IκB phosphorylation and facilitates its ubiquitination and degradation, results in NF-κB activation. Activated NF-κB then translocates to the nucleus, which results in its binding with target DNA (particularly genes that encoded pro-inflammatory factors) or cooperation with other transcription regulatory factors (25,26). Previous studies have demonstrated that various atherogenic stimuli, including oxidant stress, inflammatory responses and smoking, activate the NF-κB dimer; therefore, controlling NF-κB activation is considered to be important for estimating the therapeutic abilities of anti-atherogenic drugs. In the present study, NF-κB and IκB activations were evaluated following TSIIA treatment. TSIIA decreased NF-κB activation, increased IκB levels, and reduced the expression of pro-inflammatory factors (Fig. 3). Thus, the inhibitory effects of TSIIA on NF-κB activity may partially benefit from its effective radical clearance abilities. The antioxidant abilities of TSIIA were identified in earlier studies (36,37).

RAGE and downstream MAPK signaling is significantly elevated in atherosclerosis, and this pathway is associated with NF-κB activation, inflammatory mediator generation and oxidative stress in macrophages. Furthermore, RAGE has the unique ability to sustain NF-κB activation through de novo synthesis.
of NF-κB p65 mRNA and provide a constantly growing pool of transcriptionally active NF-κB (38). Studies have demonstrated that RAGE overexpression is associated with enhanced inflammatory reactions within lesion macrophages and that this effect may contribute to plaque destabilization by inducing MMP expression (39). Furthermore, RAGE may destabilize the atherosclerotic plaques by inducing MAPK- and prosta-
glandin E2-dependent MMP-2 and MMP-9 production (40,41).

In a previous report, RAGE knockout or blockade in apoE-/- mice resulted in decreased expression of MMP and other inflammatory cytokines (10,42). MAPKs are conserved serine/threonine kinases that are part of a coordinated and integrated signal pathway that responds to diverse atherosclerotic stimuli, oxidative damage and inflammatory mediators/responses.

Furthermore, numerous studies identified that the activation of ERK and other members of the MAPK family, including p38 and JNK, subsequently activates activator protein-1 and NF-κB, and are thus pivotal for the induction of MMPs and other inflammatory factors in macrophages (43-45). Findings of several studies have indicated that the activation of ERK1/2, JNK and p38 are important signaling mechanisms responsible for the expression of inflammatory factors, including ICAM-1, VCAM-1, and MCP-1, within macrophages when RAGE is activated in vivo or in vitro (46,47). The present study identified the inhibitory effects of TSIIA on the activation of MAPK and RAGE signals, and the suppression of MMP2, 3 and 9 (Fig. 4) and inflammatory factors, including ICAM-1, VCAM-1 and MCP-1 (Fig. 3) following TSIIA treatment.

In conclusion, the current study provides novel data to support the hypothesis that TSIIA administration may stabilize vulnerable plaques, particularly due to its anti-inflammatory effects, suppression of NF-κB, MAPK proteins phosphorylation and the RAGE axis, subsequently leading to decreased MMPs-induced matrix degradation and inflammatory factor expression in apoE-/- mice.

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