Tormentic acid inhibits H_2O_2-induced oxidative stress and inflammation in rat vascular smooth muscle cells via inhibition of the NF-κB signaling pathway

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Abstract. Tormentic acid (TA) is a triterpene isolated from the stem bark of the plant *Vochysia divergens* and has been reported to exhibit anticancer, anti-inflammatory and anti-atherogenic properties. However, the functions of TA in hydrogen peroxide (H_2O_2)-induced oxidative stress and inflammation in rat vascular smooth muscle cells (RVSMCs) remain unclear. Therefore, the present study aimed to investigate whether TA suppressed H_2O_2-induced oxidative stress and inflammation in RVSMCs, and to determine its molecular mechanisms. The present study demonstrated that TA inhibited reactive oxygen species (ROS) generation, induced H_2O_2 in RVSMCs, and inhibited H_2O_2-induced expression of inducible nitric oxide synthase (iNOS) and NADPH oxidase (NOX) in RVSMCs. In addition, TA significantly decreased the production of tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and IL-1β. Furthermore, TA pretreatment prevented nuclear factor-κB (NF-κB) subunit p65 phosphorylation and NF-κB inhibitor α (IκBα) degradation induced by H_2O_2 in RVSMCs. TA is, therefore, suggested to inhibit H_2O_2-induced oxidative stress and inflammation in RVSMCs via inhibition of the NF-κB signaling pathway. TA may have potential as a pharmacological agent in the prevention or treatment of atherosclerosis.

Introduction

Atherosclerosis is a complex pathology involving several processes, including subendothelial retention of atherogenic lipoproteins, oxidative stress, inflammation and cellular proliferation (1). Vascular smooth muscle cells (VSMCs) may contribute to the development of atherosclerosis through the production of inflammatory cytokines, such as monocyte chemoattractant protein-1, and the synthesis of matrix proteins (2).

Reactive oxygen species (ROS), for example superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), are physiological and pathophysiological signaling molecules that participate in the development of atherosclerosis (3,4). Excessive production of ROS, partially through upregulation of DNA damage pathways, is a central mechanism that mediates pathological activation of VSMCs. In addition, ROS activate multiple pro-inflammatory transcription factors, including nuclear factor erythroid 2-related factor 2, nuclear factor-κB (NF-κB), and activator protein 1, which regulate the expression of adhesion molecules and chemokines in VSMCs (5). Therefore, targeting ROS is an important therapeutic strategy for atherosclerosis.

Tormentic acid (TA) is a triterpene isolated from the stem bark of the plant *Vochysia divergens*. Previous studies have demonstrated that TA has anticancer, anti-oxidant, anti-inflammatory and hypoglycemic properties (6-9). TA was demonstrated to suppress high-fat diet-induced diabetes and hyperlipidemia via glucose transporter 4 and adenosine monophosphate-activated protein kinase phosphorylation (10). Fogo *et al* (11) previously reported that TA significantly reduced VSMC proliferation and survival. In addition, TA inhibited lipopolysaccharide-induced inducible nitric oxide synthase (iNOS), cyclooxygenase-2, and tumor necrosis factor-α (TNF-α) expression in RAW264.7 cells (12). However, the impact of TA on H_2O_2-induced oxidative stress and inflammation in rat VSMCs (RVSMCs) remains unclear. Therefore, the aim of the present study was to investigate whether TA suppressed H_2O_2-induced oxidative stress and inflammation in RVSMCs, and to determine the molecular mechanisms.

Materials and methods

Animal and RVSMC preparation. Female Sprague Dawley (SD) rats (age, 6 weeks; weight, 180-200 g) were obtained from the Animal Breeding Center of the People's Hospital of Tianjin

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City (Tianjin, China). They were housed in barrier facilities with 12 h light/dark cycles at 22±2˚C, and had access to laboratory chow (Jiangsu Xietong Medicine Biological Engineering Co., Ltd., Jiangsu, China) and tap water *ad libitum*. After 1 week of feeding, the animals were anesthetized by subcutaneous injection of sodium pentobarbital (40 mg/kg body weight). All experiments were performed in accordance with the institutional guidelines for animal care. This study was approved by the ethics committee of the People's Hospital of Tianjin City (Tianjin, China).

RVSMCs were enzymatically isolated from the aortas of female SD rats according to the methods described in a previous study (13), and were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin and 200 mM L-glutamine in a humidified 5% CO_2 atmosphere at 37˚C. For all experiments, RVSMCs (used at passages 5-8) were cultured to 70-80% confluence and serum-starved in DMEM without FBS for 24 h.

H_2O_2-induced oxidant stress. Cells were pretreated with various concentrations of TA (12.5, 25 and 50 μM; Shaanxi Institute for Food and Drug Control, Shaanxi, China) for 2 h, followed by the addition of H_2O_2 (100 μM final concentration) for a further 24 h. Controls performed were 2 h TA pretreatment without H_2O_2 stimulation, and 24 h H_2O_2 treatment without 2 h TA pretreatment.

Cell viability assay. Cell viability was assessed by Cell Counting Kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology, Shanghai, China). In brief, RVSMCs were seeded into 96-well plates at $1x10^4$ cells per well and cultured for 24 h to adhere. Following the described H_2O_2 and TA treatments, 10 μl CCK-8 reagent was added to each well and the cells incubated for a further 2 h. Finally, the absorbance was read at 570 nm (A_{570}) using a Bio-Rad enzyme-linked immunosorbent assay (ELISA) microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The viability of cells was calculated as (A_{570} of treated groups/A_{570} of control group) x100%.

Measurement of cellular ROS levels. RVSMCs were stained with 5 μM dihydroethidium (DHE; Molecular Probes; Thermo Fisher Scientific, Inc.) for 30 min at 37˚C. Fluorescence of DHE was measured with a fluorescence microscope (excitation wavelength 488 nm and emission wavelength 585 nm), quantified using ImageJ software (version, 1.46; National Institutes of Health, Bethesda, MD, USA).

ELISA assay. Following RVSMC incubation with TA (12.5, 25 and 50 μM) for 24 h, the cells were exposed to H_2O_2 for a further 2 h. Samples of the supernatant were collected from each well to measure TNF-α (cat. no. RAB0479; Sigma-Aldrich), interleukin (IL)-6 (cat. no. 10406; Sigma-Aldrich) and IL-1β (cat. no. RAB0278; Sigma-Aldrich) levels by ELISA.

Western blot analysis. Cell lysate was prepared from RVSMCs using lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). Equal amounts of protein samples (30 μg total protein per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). After blocking with 2% non-fat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature, the membranes were probed with the following primary antibodies in TBS plus 0.1% Tween-20 (TBST) containing 5% BSA (Sigma-Aldrich; Merck Millipore) at 4˚C overnight: Mouse anti-rabbit nitric oxide synthase (NOS; cat. no. N7782; dilution, 1:1,000; Sigma-Aldrich; Merck Millipore); mouse anti-rabbit nicotinamide adenine dinucleotide phosphate (NADPH) -oxidase 1 (NOX1; cat. no. SAB2108601; dilution, 1:2,000; Sigma-Aldrich; Merck Millipore); mouse anti-rabbit neutrophil cytosolic factor 1 (p47phox; cat. no. sc-14015; dilution, 1:1,500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); and mouse anti-rabbit glyceraldehyde 3-phosphate dehydrogenase (GAPDH; cat. no. sc-25778; dilution, 1:1,500; Santa Cruz Biotechnology, Inc.). Membranes were then washed three times in TBST for 5 min per wash before they were incubated with goat anti-rabbit horseradish peroxidase-conjugated anti-IgG secondary antibody (cat. no. sc-2054; dilution, 1:3,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Immune complexes were visualized using enhanced chemiluminescence reagent (Gibco; Thermo Fisher Scientific, Inc.). Developed films were scanned, and the optical densities were analyzed using ImageJ software (version, 1.37; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical differences were analyzed using one-way analysis of variance, followed by Dunnett's multiple comparison post-hoc test. Differences in the cumulative clinical score were analyzed using the non-parametric Mann-Whitney test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of TA on RVSMC viability. The effect of TA on cell viability in H_2O_2-induced RVSMCs was examined by CCK-8 assay. The significant decrease in cell viability resulting from H_2O_2 treatment ($P=0.017$) compared with untreated control; Fig. 1A), was significantly attenuated by TA in a dose-dependent manner ($P<0.05$; Fig. 1A). To exclude any proliferative effect of TA from analysis, cell viability was assessed following TA treatment alone, and was demonstrated to be unaffected by treatment with TA at any of the concentrations tested (12.5, 25, and 50 μM) compared with untreated control (Fig. 1B). Thus, the concentrations 12.5, 25 and 50 μM were used in subsequent experiments.

Effect of TA on ROS generation in RVSMCs exposed to H_2O_2. As increased ROS levels, resulting in oxidative stress, are considered to be important in the pathogenesis of atherosclerosis, the effect of TA on ROS generation in RVSMCs exposed to H_2O_2 was investigated. Treatment with H_2O_2 for 2 h significantly increased the production of ROS compared with untreated control cells ($P=0.019$; Fig. 2). However, pretreatment with 25 μM ($P=0.041$) and 50 μM TA ($P=0.024$) significantly inhibited ROS generation compared with cells treated with H_2O_2 only (Fig. 2).

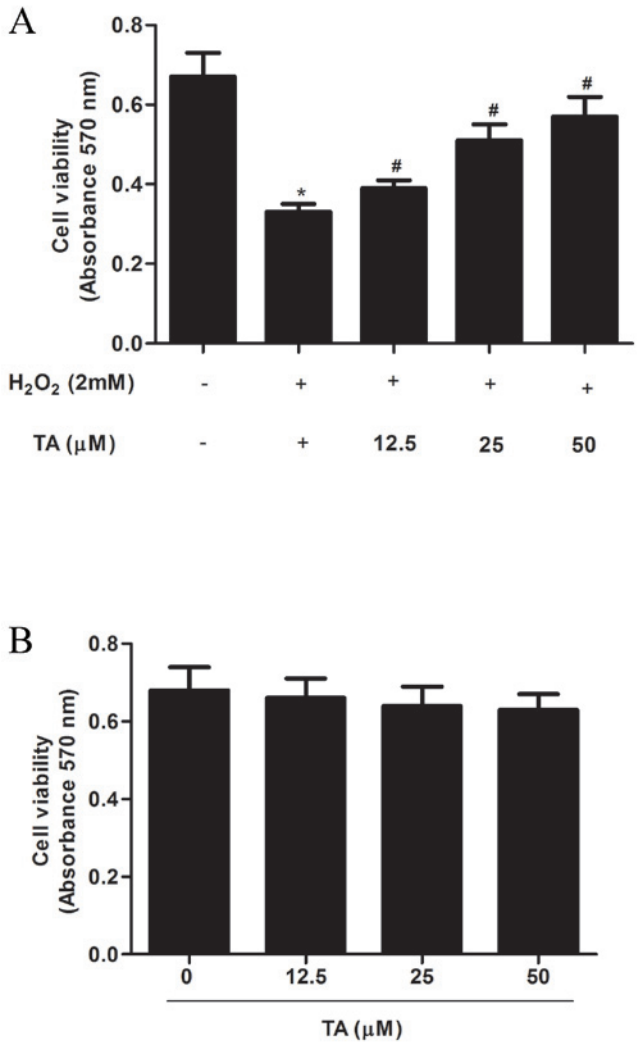

Figure 1. Effect of TA on RVSMC viability. (A) RVSMC viability was determined by CCK-8 assay following pretreatment with various concentrations of TA (0, 12.5, 25 and 50 μM) for 2 h and H_2O_2 treatment (final concentration 100 μM) for a further 24 h. (B) RVSMC viability was determined by CCK-8 assay following pretreatment with various concentrations of TA (0, 10, 50 and 100 μM) for 24 h. Data are presented as the mean ± standard deviation obtained from five individual experiments performed in triplicate. *P<0.05 vs. untreated control; #P<0.05 vs. H_2O_2-treated control. TA, tormentic acid; RVSMC, rat vascular smooth muscle cell; CCK, cell counting kit; H_2O_2, hydrogen peroxide.

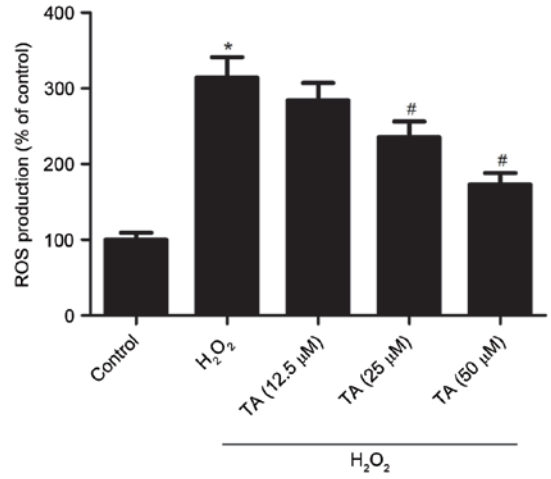

Figure 2. Effect of TA on ROS generation in RVSMCs exposed to H_2O_2. The level of intracellular ROS was measured following RVSMC 2 h pretreatment with various concentrations of TA (12.5, 25 and 50 μM) and H_2O_2 treatment (final concentration 100 μM) for a further 24 h. Data are presented as the mean ± standard deviation obtained from five individual experiments performed in triplicate. *P<0.05 vs. untreated control; #P<0.05 vs. H_2O_2-treated control. ROS, reactive oxygen species; TA, tormentic acid; H_2O_2, hydrogen peroxide; RVSMC, rat vascular smooth muscle cell.

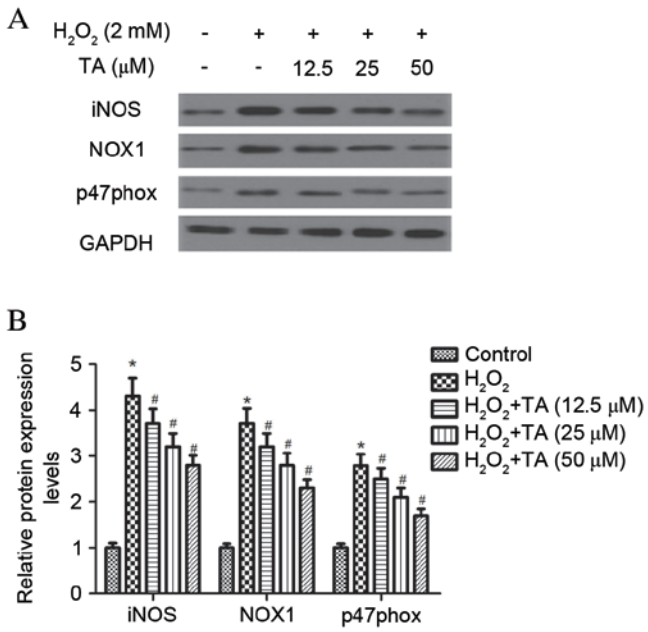

Figure 3. Effect of TA on iNOS and NADPH oxidase expression in RVSMCs exposed to H_2O_2. RVSMCs were pretreated with various concentrations of TA (0, 12.5, 25 and 50 μM) for 2 h, followed by the addition of H_2O_2 (100 μM as final concentration) for another 24 h. (A) Proteins were subjected to western blot analysis and (B) the relative protein levels of iNOS, p47phox and NADPH oxidase 1 were analyzed, using GAPDH as the loading control. Data are presented as the mean ± standard deviation obtained from five individual experiments performed in triplicate. *P<0.05 vs. untreated control; #P<0.05 vs. H_2O_2-treated control. RVSMC, rat vascular smooth muscle cell; TA, tormentic acid; iNOS, inducible nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; H_2O_2, hydrogen peroxide; NOX1, NADPH oxidase 1; p47phox, neutrophil cytosolic factor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Effect of TA on iNOS, NOX1 and p47phox protein expression in RVSMCs exposed to H_2O_2. The effect of TA on iNOS, NOX1 and p47phox protein expression levels were evaluated by western blot analysis in RVSMCs exposed to H_2O_2. Treatment with H_2O_2 for 2 h significantly increased the protein expression levels of iNOS (P=0.017), NOX1 (P=0.026) and p47phox (P=0.031) compared with untreated control cells (Fig. 3A and B). However, pretreatment with TA significantly inhibited the H_2O_2-induced expression of all three proteins in RVSMCs in a dose-dependent manner, compared with cells treated with H_2O_2 only (P<0.05; Fig. 3A and B).

Effect of TA on TNF-α, IL-6 and IL-1β production in RVSMCs induced with H_2O_2. To investigate the anti-inflammatory effects of TA on RVSMCs, TNF-α, IL-6 and IL-1β production was evaluated using ELISA, revealing that H_2O_2 significantly increased the production of TNF-α (P=0.021; Fig. 4A), IL-6

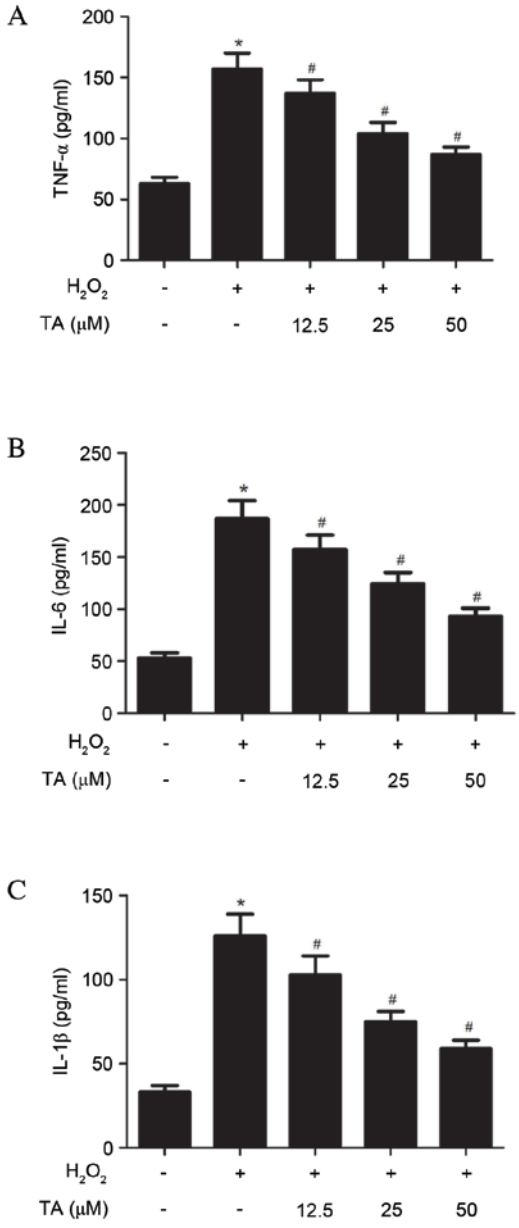

Figure 4. Effects of TA on TNF-α, IL-6 and IL-1β production in RVSMCs induced with H_2O_2. RVSMCs were pretreated with various concentrations of TA (0, 12.5, 25 and 50 μM) for 2 h, followed by treatment with H_2O_2 (final concentration 100 μM) for a further 24 h. Enzyme-linked immunosorbent assay was performed to quantify (A) TNF-α, (B) IL-6 and (C) IL-1β levels. Data are presented as the mean ± standard deviation obtained from five individual experiments performed in triplicate. *P<0.05 vs. untreated control; #P<0.05 vs. H_2O_2-treated control. RVSMC, rat vascular smooth muscle cell; TNF-α, tumor necrosis factor-α; H_2O_2, hydrogen peroxide; TA, tormentic acid; IL, interleukin.

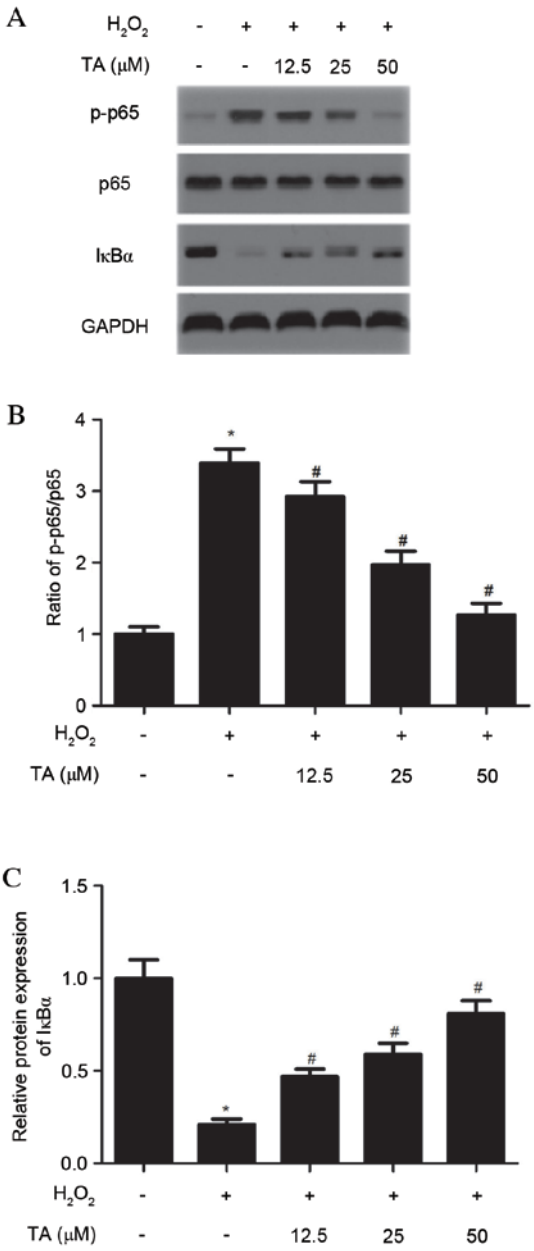

Figure 5. Effects of TA on the NF-κB signaling pathway in H_2O_2-induced RVSMCs. RVSMCs were pretreated with various concentrations of TA (0, 12.5, 25 and 50 μM) for 2 h, followed by the addition of H_2O_2 (final concentration 100 μM) for a further 24 h. (A) Proteins were subjected to western blot analysis to detect relative protein levels of NF-κB p65, p-NF-κB p65 and IκBα, using GAPDH as the loading control. For each treatment, (B) the ratio of phosphorylated to non-phosphorylated NF-κB p65, and (C) the relative protein levels of IκBα were analyzed. Data are presented as the mean ± standard deviation obtained from five individual experiments performed in triplicate. *P<0.05 vs. untreated control; #P<0.05 vs. H_2O_2-treated control. RVSMC, rat vascular smooth muscle cell; NF-κB, nuclear factor-κB; H_2O_2, hydrogen peroxide; TA, tormentic acid; p-, phosphorylated; IκBα, NF-κB inhibitor-α.

(P=0.016; Fig. 4B) and IL-1β (P=0.031; Fig. 4C) in the RVSMCs, compared with untreated cells. Compared with cells treated with H_2O_2 only, TA significantly decreased the production of TNF-α (P=0.028; Fig. 4A), IL-6 (P=0.023; Fig. 4B) and IL-1β (P=0.016; Fig. 4C) in a dose-dependent manner.

Effects of TA on NF-κB signaling pathway in H_2O_2-induced RVSMCs. As NF-κB has been previously reported to be important in the regulation of cytokine production, the effects of TA on H_2O_2-induced NF-κB activation were investigated. As demonstrated in Fig. 5, H_2O_2 significantly increased NF-κB p65 phosphorylation (P=0.018; Fig. 5A and B) and IκBα degradation (P=0.027; Fig. 5A and C) compared with untreated cells. However, TA pretreatment prevented NF-κB p65 phosphorylation (P=0.013; Fig. 5A and B) and IκBα degradation (P=0.019; Fig. 5A and C) induced by H_2O_2 in RVSMCs

in a dose-dependent manner, compared with cells treated with H_2O_2 only. No differences in the levels of NF-κB p65 were observed in H_2O_2-induced RVSMC (Fig. 5A; quantitative data not shown).

Discussion

The present study demonstrated that TA inhibits H_2O_2-induced ROS generation in RVSMCs, and H_2O_2-induced expression of iNOS and NOX1 in RVSMCs. In addition, TA was demonstrated to significantly decrease the production of TNF-α, IL-6 and IL-1β. Furthermore, TA pretreatment reduced NF-κB p65 phosphorylation and IκBα degradation induced by H_2O_2 in RVSMCs.

Oxidative stress is frequently involved in cardiovascular disease and is a common feature of early stage-atherosclerosis as a response to vascular injury (14,15). H_2O_2 has previously been demonstrated to activate signaling pathways to stimulate ROS production in vascular cells (16-18). The present study demonstrated that treatment with TA significantly inhibits the generation of ROS induced by H_2O_2 in RVSMCs.

Previous studies have reported that iNOS may exacerbate atherosclerosis, as $ApoE^{-/-}$ mice lacking the iNOS gene exhibit decreased atherosclerotic lesion formation compared with $ApoE^{-/-}$ mice (19). NOXs are transmembrane enzymes that transport electrons from cytoplasmic NADPH to molecular oxygen, leading to superoxide generation, and are therefore an important source of vascular ROS (20). Several studies have demonstrated that NOX1 expression is increased in atherosclerosis (21,22), and that p47phox is an essential component of NOX (23). In smooth muscle cells, H_2O_2 activates NOX, resulting in the production of O_2^-, and, consequently, oxidant-induced injury (24). Similarly, the present study observed that H_2O_2 significantly increases the expression of iNOS, NOX1 and p47phox in RVSMCs, whereas pretreatment with TA significantly abrogates this effect.

Inflammatory cytokines are involved in the early stages of atherosclerosis (25). TNF-α is the earliest and primary endogenous mediator in the process of inflammation, and is involved in promoting inflammatory cell infiltration, injuring vascular endothelial cells and stimulating the generation of ROS (26). IL-1β is one of the most potent pro-inflammatory cytokines and endogenous pyrogens, and stimulates the acute phase response (27). In response to oxidative stimuli, VSMCs undergo a phenotypic change to a 'proliferative, migrating and synthetic' state, characterized by excess extracellular matrix and inflammatory cytokine production (28). In accordance with these results, the present study demonstrated that H_2O_2 significantly increases the production of TNF-α, IL-6 and IL-1β in RVSMCs. However, TA significantly attenuated H_2O_2-induced production of TNF-α, IL-6 and IL-1β in RVSMCs.

NF-κB represents a family of transcription factors, including p50 and p65 that are important in the regulation of inflammatory responses (29). In response to oxidative stress, Activated IκB kinase phosphorylates the NF-κB inhibitor, IκB, resulting in its polyubiquitination and proteasomal degradation (30). IκB degradation leads to the translocation of NF-κB p50 and p65 to the nucleus, which results in the transcription of a variety of genes participating in diverse cellular processes, including inflammation, proliferation, apoptosis, and cellular senescence (31). Pierce *et al* (32) observed that the NF-κB inhibitor, salsalate, increases IκB expression levels, and decreases the levels of NF-κB and p47phox NADPH oxidase subunit in endothelial cells. The activation of NF-κB by ROS has also previously been demonstrated to induce TNF-α, IL-6 and IL-1β release in VSMCs (33). The present study demonstrated that pretreatment of RVSMCs with TA prevents H_2O_2-induced NF-κB p65 phosphorylation and IκBα degradation in a dose-dependent manner. This suggests that TA may reduce H_2O_2-induced ROS generation through the action of NOX, and reduces TNF-α, IL-6 and IL-1β protein expression levels and induction of iNOS through inhibition of NF-κB signaling activation.

In conclusion, the present study demonstrated that TA inhibits H_2O_2-induced oxidative stress and inflammation in RVSMCs via inhibition of the NF-κB signaling pathway. TA may, therefore, have potential as a pharmacological agent in the prevention or treatment of atherosclerosis.

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