

Myricitrin inhibits vascular adhesion molecule expression in TNF- α -stimulated vascular smooth muscle cells

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Abstract. Increased expression of adhesion molecules is thought to serve an important role in the pathogenesis of atherosclerosis. Myricitrin, a bioactive compound of *Myrica cerifera*, has been demonstrated to exhibit anti-atherogenic effects. However, the effect of myricitrin on the expression of adhesion molecules in vascular smooth muscle cells (VSMCs) remains unknown. Therefore, the aim of the present study was to evaluate the inhibitory effects of myricitrin on tumor necrosis factor- α (TNF- α)-induced expression of adhesion molecules in VSMCs *in vitro*. The results revealed that myricitrin inhibited the adhesion of human THP-1 monocyte cells to TNF- α -stimulated mouse MOVAS-1 VSMC cells, and reduced the expression of adhesion molecules in TNF- α -stimulated MOVAS-1 cells. In addition, myricitrin significantly inhibited the TNF- α -induced expression of nuclear factor (NF)- κ B p65, and prevented the TNF- α -induced degradation of nuclear factor of κ light chain enhancer in B-cells inhibitor α . Furthermore, myricitrin inhibited the production of intracellular reactive oxygen species in TNF- α -stimulated MOVAS-1 cells. In conclusion, the results of the present study indicated that myricitrin inhibits the expression of vascular cell adhesion protein-1 and intercellular adhesion molecule-1 in TNF- α -stimulated MOVAS-1 cells potentially via the NF- κ B signaling pathway. Therefore, myricitrin may be an effective pharmacological agent for the prevention or treatment of atherosclerosis.

Introduction

Atherosclerosis is the most common type of coronary artery disease and the leading cause of morbidity and mortality worldwide (1). It is characterized by the accumulation of lipids

in the arterial vessel wall (2). A number of studies have demonstrated that the expression of cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), is increased in vascular smooth muscle cells (VSMCs), leading to increased neointima or atherosclerotic lesion formation (3-5). In addition, the expression of adhesion molecules in vascular cells is affected by inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) (6). Therefore, a promising therapeutic approach for the treatment of pathological inflammation may be to reduce the expression of adhesion molecules in VSMCs.

Myricitrin, a bioactive compound of *Myrica cerifera*, has been demonstrated to exhibit a number of pharmacological actions, including antinociceptive, anti-inflammatory, anti-cancer and anti-oxidative activities (7-9). Domitrović *et al* (10) reported that myricitrin significantly reduced the carbon tetrachloride-induced increase in cyclooxygenase-2 and TNF- α levels in the liver. In addition, a previous study demonstrated that myricitrin exhibits anti-atherogenic effects (11). Administration of myricitrin *in vivo* decreased the vascular wall thickness of the aortic arch in apolipoprotein E^{-/-} mice, and myricitrin treatment significantly attenuated oxidized low-density lipoprotein-induced endothelial cell apoptosis (11). However, the effect of myricitrin on the expression of adhesion molecules in VSMCs remains unknown. Therefore, the aim of the present study was to evaluate the inhibitory effects of myricitrin on adhesion molecule expression induced by TNF- α in VSMCs *in vitro*.

Materials and methods

Cell culture. The VSMC cell line MOVAS-1 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Sigma; Merck KGaA, Darmstadt, Germany), 100 μ g/ml streptomycin (Sigma; Merck KGaA), and 200 mM L-glutamine (Sigma; Merck KGaA) in a humidified 5% CO₂ atmosphere at 37°C. THP-1 cells (ATCC) were maintained in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin in an incubator with a humidified atmosphere of 5% CO₂ at 37°C.

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Cell adhesion assay. The cell adhesion assay was performed as previously described (12). Briefly, MOVAS-1 cells were plated in 96-well plates at a density of $\sim 1 \times 10^4$ cells/well, which were then pretreated with varying concentrations (2.5, 5 and 10 μM) of myricitrin (Sigma; Merck KGaA) for 2 h, followed by stimulation with or without TNF- α (10 ng/ml; Sigma; Merck KGaA) for 8 h. The media was removed from the wells and 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-labeled (5 μM ; Sigma; Merck KGaA) THP-1 cells (1×10^5 cells/ml) in 0.2 ml medium were added to each well. Following incubation for 1 h at 37°C in 5% CO₂, the wells were washed three times with 0.2 ml medium, and the number of adherent cells in 4 high power fields of view were observed using a Nikon Eclipse E600 fluorescence microscope at x100 magnification (Nikon Corporation, Tokyo, Japan).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from MOVAS-1 cells (1×10^4 cells/well) with TRIzol reagent (Abcam, Cambridge, UK) according to the manufacturer's instructions. Total RNA (5 μg) was reverse transcribed into cDNA using an oligo-(dT) primer and M-MLV reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) for qPCR analysis. qPCR was performed in a final volume of 10 μl , which consisted of 5 μl SsoFast™ EvaGreen Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 1 μl cDNA (1:50 dilution) and 2 μl each of the forward and reverse primers (1 mM). The specific primers (Invitrogen; Thermo Fisher Scientific, Inc.) were as follows: VCAM-1 sense, 5'-CAAAGG TGGATCAGATTCAAG-3' and antisense, 5'-GGTGAGCAT TATCACCCAGAA-3'; ICAM-1 sense, 5'-CAAAGGTGGATC AGATTCAAG-3' and antisense, 5'-GGTGAGCATTATCAC CCAGAA-3'; GAPDH sense, 5'-CAAAGGTGGATCAGA TTCAAG-3' and antisense, 5'-GGTGAGCATTATCACCCA GAA-3'. The thermal cycling procedure was as follows: 95°C for 4 min, followed by 40 cycles of 95°C for 25 sec, 55°C for 30 sec and 72°C for 20 sec with 2 sec for plate reading, and melting curve analysis from 65 to 95°C. GAPDH was used as the control for normalizing gene expression. The relative quantification of the gene of interest was determined using the comparative ΔC_q method (13).

Western blot analysis. MOVAS-1 cells (1×10^6 cells) were harvested, washed twice with phosphate-buffered saline, and lysed in radioimmunoprecipitation assay buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing 100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 1 mM EDTA, 10 mM β -glycerophosphate, 2 mM sodium vanadate and protease inhibitors (Cell Signaling Technology, Inc.) on ice for 10 min. Following 15 min, 0.5% Nonidet P (NP)-40 (Mairybio; Beijing Minhai Biotechnology Co., Ltd., Beijing, China) was added to lyse the cells, which were vortexed for 10 sec. Then, cytosolic cell extracts were obtained following centrifugation at 1,500 x g for 10 min at 4°C. The collected nuclei were re-suspended in 50 μl of Buffer C [20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM PMSF and Protease Inhibitor Cocktail] and were then incubated on ice for 20 min with intermittent agitation. Nuclear cell extracts were obtained following centrifugation for 10 min at 13,000 x g and 4°C. Equal amounts (30 μg) of

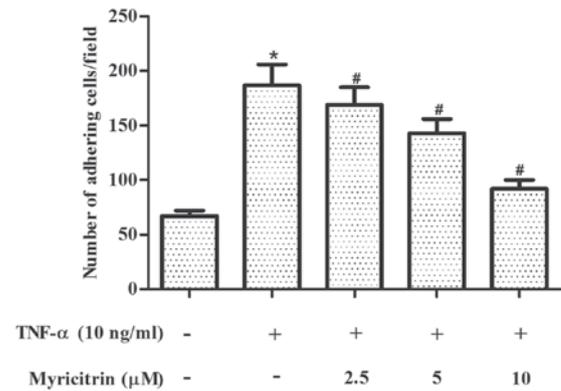


Figure 1. Inhibition of THP-1 cell adhesion to TNF- α -stimulated MOVAS-1 cells by myricitrin. Confluent MOVAS-1 cells were pretreated for 2 h with 0, 2.5, 5 or 10 μM myricitrin. The cells were then washed twice with medium and incubated with TNF- α (10 ng/ml) for 8 h. The 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-labeled THP-1 cells were added to the MOVAS-1 cell monolayer and allowed to adhere for 1 h. Adherent THP-1 to MOVAS-1 cells were observed under a fluorescence microscope at x100 magnification. All experiments were repeated at least three times. Data are presented as the mean \pm standard deviation. *P<0.05 vs. the untreated control group, #P<0.05 vs. the TNF- α -only treated group. TNF- α , tumor necrosis factor- α .

protein sample were separated using a 10% SDS-PAGE gel and then electrotransferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking in Tris-buffered saline buffer (50 mmol/l NaCl, 10 mmol/l Tris, pH 7.4) containing 5% non-fat milk for 1 h at room temperature, the membranes were then incubated with the appropriate primary antibodies against VCAM-1 (1:3,000; cat. no. sc-13160), ICAM-1 (1:3,000; cat. no. sc-8439), nuclear factor (NF)- κB p65 (1:2,000; cat. no. sc-8008), nuclear factor of κ light chain gene enhancer in B-cells inhibitor α (I κ B α ; 1:2,500; cat. no. sc-1643) and GAPDH (1:3,000; cat. no. sc-47724) at 4°C overnight, followed by incubation with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:3,000; cat. no. sc-2005; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. The membranes were exposed and visualized using enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.). Densitometry was performed using Gel-Pro Analyzer software version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Measurement of intracellular reactive oxygen species (ROS) accumulation. Intracellular ROS levels were measured using dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich; Merck KGaA,) staining. Briefly, MOVAS-1 cells (1×10^4 cells/well) treated with myricitrin in the presence or absence of TNF- α (as described in the *Cell adhesion assay* section) were incubated with 5 μM DCFH-DA for 30 min at 37°C in the dark, and were then washed with serum-free medium three times. The fluorescence intensity was measured at an excitation and emission wavelength of 485 and 520 nm, respectively, using a fluorescence spectrophotometer (Infinite M1000; Tecan Austria GmbH, Grödig, Austria). The ROS level was expressed as units of fluorescence.

Statistical analysis. The results were analyzed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA) and data are expressed as the mean \pm standard deviation. Statistical

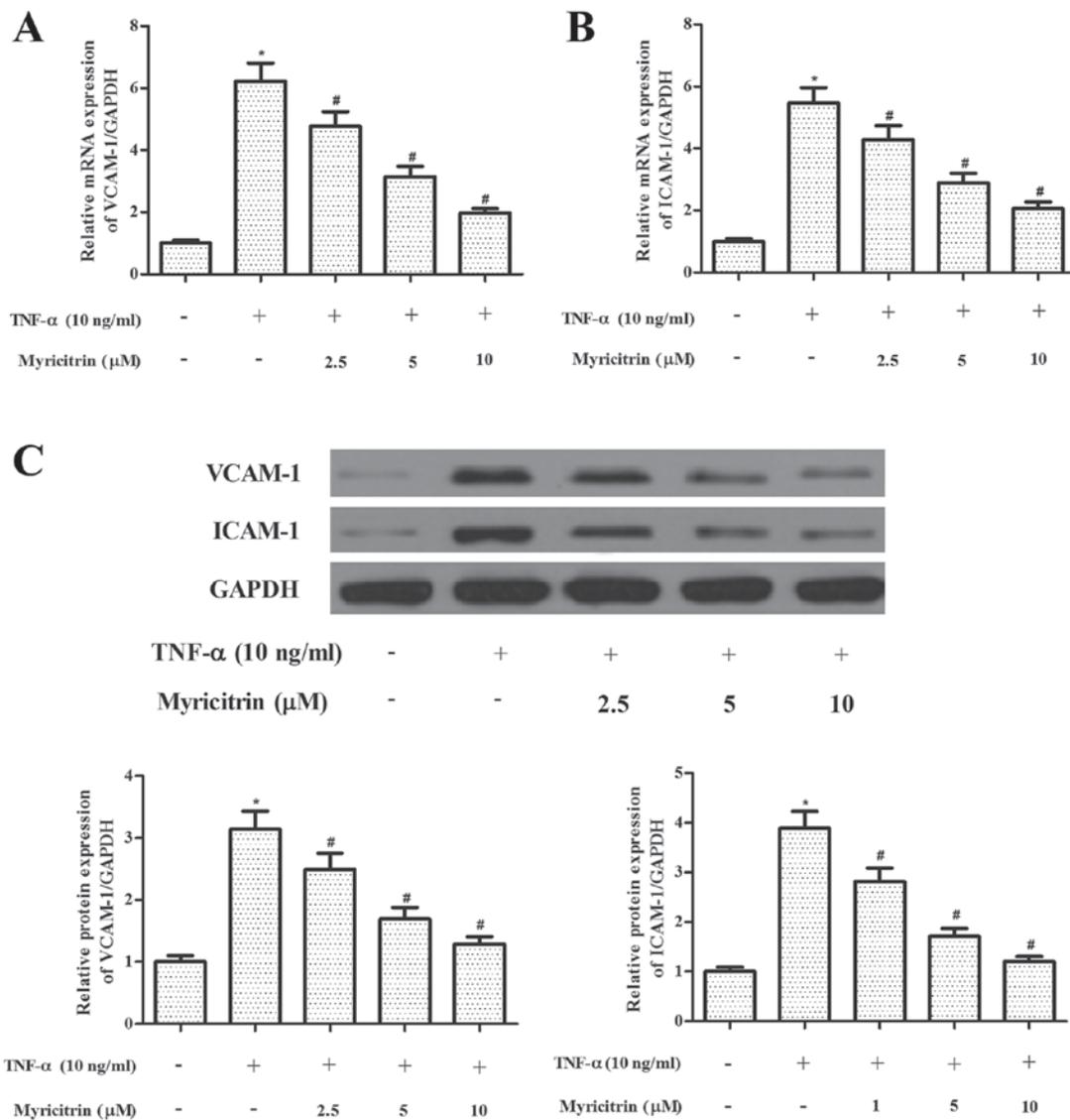


Figure 2. Effect of myricitrin on adhesion molecule expression in TNF- α -stimulated MOVAS-1 cells. MOVAS-1 cells were preincubated with varying concentrations (0, 2.5, 5 and 10 μ M) of myricitrin for 2 h and stimulated with TNF- α (10 ng/ml) for 8 h. (A) VCAM-1 and (B) ICAM-1 mRNA expression was determined by reverse transcription-quantitative polymerase chain reaction analysis. (C) VCAM-1 and ICAM-1 protein expression was determined by western blot analysis. All experiments were repeated at least three times. Data are presented as mean \pm standard deviation. *P<0.05 vs. the untreated control group, #P<0.05 vs. the TNF- α -only treated group. TNF- α , tumor necrosis factor- α ; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1.

significance was assessed by one-way analysis of variance followed by a Tukey post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Myricitrin inhibited the adhesion of THP-1 cells to TNF- α -stimulated MOVAS-1 cells. The effect of myricitrin on the adhesion of THP-1 cells to MOVAS-1 cells in response to TNF- α was first evaluated. As shown in Fig. 1, treatment of confluent MOVAS-1 cells with TNF- α for 8 h resulted in a 2.8-fold increase in the adhesion of THP-1 monocytic cells when compared with untreated MOVAS-1 cells (P<0.05). By contrast, pretreatment with myricitrin significantly inhibited the adhesion of THP-1 cells to TNF- α -stimulated MOVAS-1 cells in a dose-dependent manner (2.5 μ M, P<0.05; 5 μ M, P<0.05; 10 μ M, P<0.05; Fig. 1).

Myricitrin inhibited the expression of adhesion molecules in TNF- α -stimulated MOVAS-1 cells. The increased expression of adhesion molecules is thought to serve an important role in the pathogenesis of atherosclerosis (4). Therefore, the present study investigated the effect of myricitrin on the expression of adhesion molecules in MOVAS-1 cells in response to TNF- α treatment. The RT-qPCR analysis results revealed that TNF- α significantly increased the mRNA expression levels of VCAM-1 and ICAM-1 in MOVAS-1 cells when compared with untreated controls. However, myricitrin significantly suppressed the mRNA expression levels of VCAM-1 and ICAM-1 in TNF- α -stimulated MOVAS-1 cells (VCAM-1, P<0.05; ICAM-1, P<0.05; Fig. 2A and B). In addition, myricitrin significantly suppressed the protein expression levels of VCAM-1 and ICAM-1 in TNF- α -stimulated MOVAS-1 cells (P<0.05 and P<0.05, respectively; Fig. 2C).

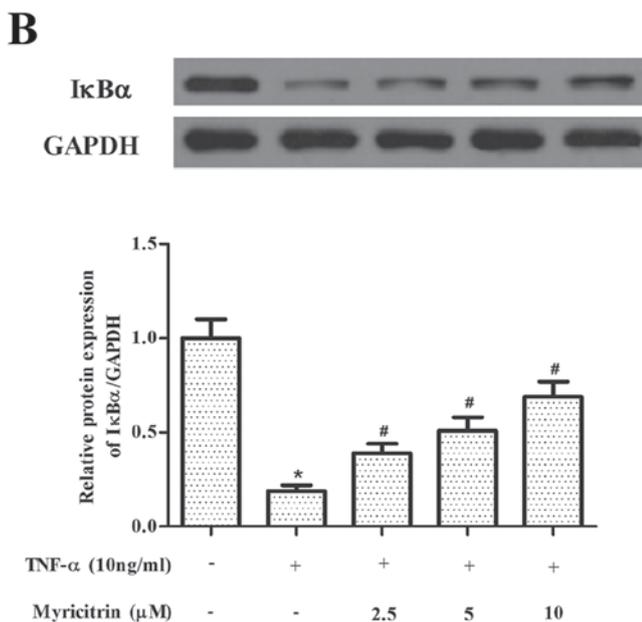
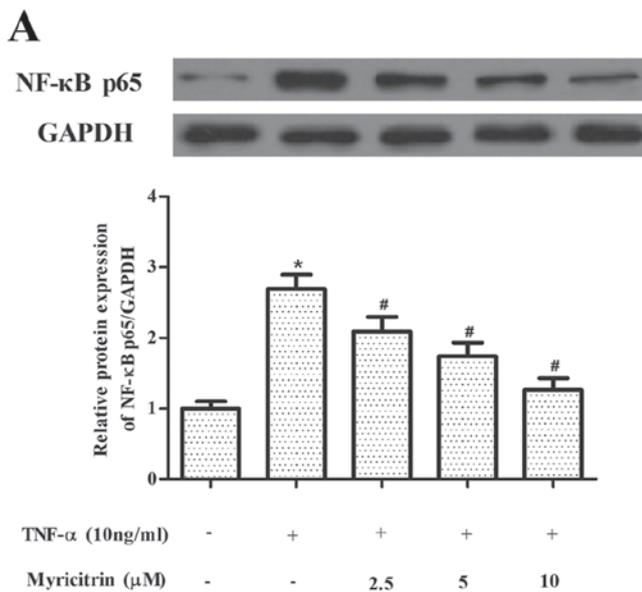


Figure 3. Effect of myricitrin on NF- κ B activation and I κ B α degradation in TNF- α -stimulated MOVAS-1 cells. MOVAS-1 cells were preincubated with 0, 2.5, 5 or 10 μ M of myricitrin for 2 h, before they were treated with TNF- α for 8 h. (A) The nuclear level of NF- κ B p65 was detected by western blotting to analyze the translocation of NF- κ B. (B) I κ B α degradation as determined by western blot analysis. All experiments were repeated at least three times. Data are presented as the mean \pm standard deviation. *P<0.05 vs. the untreated control group, #P<0.05 vs. the TNF- α -only treated group. NF- κ B, nuclear factor κ B; I κ B α , nuclear factor of κ light chain gene enhancer in B-cells inhibitor α ; TNF- α , tumor necrosis factor α .

Myricitrin suppressed TNF- α -induced NF- κ B activation in MOVAS-1 cells. NF- κ B has been reported to serve a critical role in the regulation of adhesion molecule expression (14). Therefore, the present study investigated the effects of myricitrin on NF- κ B activation in MOVAS-1 cells in response to TNF- α treatment. As indicated in Fig. 3, TNF- α significantly increased NF- κ B p65 protein expression and I κ B α degradation (NF- κ B, P<0.05; I κ B α , P<0.05). However, myricitrin pretreatment significantly prevented the TNF- α -induced increase in NF- κ B p65 and the TNF- α -induced degradation of I κ B α in

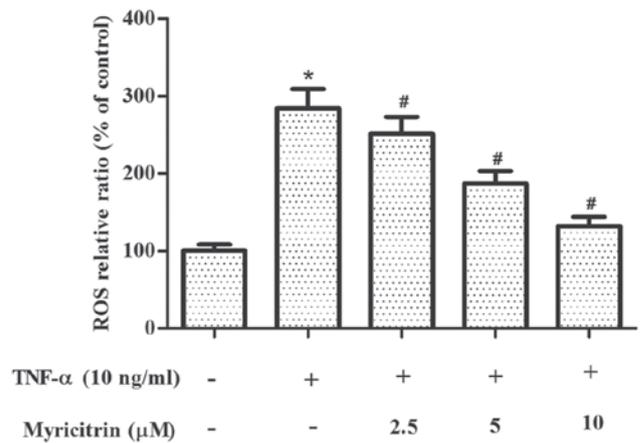


Figure 4. Effect of myricitrin on the production of ROS in TNF- α -stimulated MOVAS-1 cells. MOVAS-1 cells were treated with 0, 2.5, 5 or 10 μ M myricitrin for 2 h followed by stimulation with TNF- α (10 ng/ml) for 8 h. ROS production was measured by fluorescence spectrophotometry. All experiments were repeated at least three times. Data are presented as the mean \pm standard deviation. *P<0.05 vs. the untreated control group, #P<0.05 vs. the TNF- α -only treated group. ROS, reactive oxygen species; TNF- α , tumor necrosis factor α .

MOVAS-1 cells in a dose-dependent manner (NF- κ B, P<0.05 at 2.5, 5 and 10 μ M myricitrin; I κ B α , P<0.05 at 2.5, 5 and 10 μ M myricitrin; Fig. 3).

Myricitrin inhibited ROS production in TNF- α -stimulated MOVAS-1 cells. ROS is involved in the development of atherosclerosis (15). Therefore, the present study examined the effect of myricitrin on ROS production in MOVAS-1 cells in response to TNF- α . As shown in Fig. 4, treatment with TNF- α significantly increased the production of ROS when compared to that of the untreated control cells (P<0.05). By contrast, pretreatment with myricitrin significantly inhibited TNF- α -induced ROS production in MOVAS-1 cells in a dose-dependent manner (2.5 μ M, P<0.05; 5 μ M, P<0.05; 10 μ M, P<0.05; Fig. 4).

Discussion

To the best of the authors' knowledge, the present study is the first to delineate the effects of myricitrin on the expression of adhesion molecules in TNF- α -stimulated MOVAS-1 cells. The results indicated that myricitrin inhibits the adhesion of THP-1 cells to TNF- α -stimulated MOVAS-1 cells, as well as the expression of adhesion molecules. The underlying mechanism may involve the NF- κ B signaling pathway.

Leukocyte adhesion to VSMCs during atherosclerotic progression is primarily mediated by cell adhesion molecules, such as VCAM-1 and ICAM-1 (16). It has been demonstrated that VCAM-1 and ICAM-1 are expressed by VSMCs and are prominent in the fibrous caps of advanced atherosclerotic plaques (17). In addition, a number of studies have reported increased expression of VCAM-1 and ICAM-1 in coronary atherosclerotic tissues (18,19). Furthermore, inflammatory cytokines, such as interleukin-1 β and TNF- α , may induce the expression of VCAM-1 and ICAM-1 in VSMCs (20-22). In accordance with previous reports, the present study demonstrated that TNF- α significantly increased the adhesion of

THP-1 cells to MOVAS-1 cells and the expression of VCAM-1 and ICAM-1 in MOVAS-1 cells. By contrast, myricitrin inhibited the adhesion of THP-1 cells to TNF- α -stimulated MOVAS-1 cells, as well as the expression of VCAM-1 and ICAM-1. These results indicate that myricitrin may exhibit an inhibitory effect on the expression of adhesion molecules in TNF- α -stimulated MOVAS-1 cells.

The NF- κ B signaling pathway serves a critical role in the regulation of adhesion molecule expression (23,24). Translocation of NF- κ B to the nucleus is preceded by the phosphorylation, ubiquitination and proteolytic degradation of I κ B α (25). It has been reported that activation of the NF- κ B transcription factor by TNF- α is required for the transcriptional activation of muscle cell adhesion molecules (26,27). Furthermore, a previous study reported that oral treatment with myricitrin induced anti-inflammatory effects in dextran sulfate sodium-induced acute colitis in mice by inhibiting Akt/phosphatidylinositol-3 kinase-dependent phosphorylation and the NF- κ B signaling pathway (28). In addition, myricitrin was able to prevent the formation of advanced glycation end products by suppressing NF- κ B activation and translocation triggered by methylglyoxal in SH-SY5Y cells (29). Similarly, myricitrin pretreatment prevented NF- κ B p65 activation and I κ B α degradation in TNF- α -stimulated MOVAS-1 cells in the present study. Therefore, these data indicate that the inhibitory effect of myricitrin on the expression of adhesion molecules may be mediated, in part, through suppression of the NF- κ B signaling pathway.

ROS, which are synthesized by nicotinamide adenine dinucleotide phosphate oxidase, serve as secondary messengers that activate a number of signaling pathways, including the NF- κ B pathway (30). Activation of these signaling cascades leads to the induction of various genes that serve critical roles in the development of atherosclerosis (31). In addition, it has been reported that TNF- α induces ROS production in VSMCs (20,32,33). The present study demonstrated that TNF- α significantly increased the production of ROS in MOVAS-1 cells. By contrast, pretreatment with myricitrin significantly inhibited ROS production in a concentration-dependent manner. These results indicate that myricitrin may inhibit the activation of NF- κ B via suppression of ROS production in TNF- α -stimulated MOVAS-1 cells.

In conclusion, myricitrin inhibited the expression of VCAM-1 and ICAM-1 in TNF- α -stimulated MOVAS-1 cells potentially via the NF- κ B signaling pathway. Therefore, myricitrin may be an effective pharmacological agent for the prevention or treatment of atherosclerosis.

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