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Original Article

Carvacrol protects against tumor necrosis factor-mediated inflammation in vascular smooth muscle cell through NF-κB pathway

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Abstract: Atherosclerosis is a chronic inflammatory disease associated with increased expression of adhesion molecules in vascular smooth muscle cells (VSMCs). Carvacrol, a cyclic monoterpene, exerts protective activities in a variety of pathological states including tumor growth, inflammation, and oxidative stress. Recent studies have indicated that carvacrol has a reverse effect on vascular neointimal formation. However, its detailed mechanisms require further study to provide more scientific evidence for the clinical treatment of atherosclerosis. In the present study, we investigate the effect of carvacrol on the expression of VCAM-1 and ICAM-1 induced by TNF-α in cultured VSMCs. Pretreatment of VSMCs for 2 h with carvacrol (0.1-1 μM) dose-dependently inhibited TNF-α-induced protein and mRNA expression of VCAM-1 and ICAM-1. Carvacrol also suppressed TNF-α-induced production of intracellular reactive oxygen species (ROS) and activation of p38, ERK and JNK. Furthermore, carvacrol inhibited NK-κB activation induced by TNF-α. Carvacrol inhibited TNF-α-induced IκB kinase activation, subsequent degradation of IκBα and nuclear translocation of p65 NF-κB protein level. This study suggests that carvacrol downregulates the TNF-α-mediated induction of VCAM-1 and ICAM-1 in VSMCS by inhibiting the NF-κB, MAPK signaling pathways and intracellular ROS production. Thus, carvacrol may have beneficial effects to suppress inflammation within the atherosclerotic lesion.

Keywords: Carvacrol, anti-inflammation, cell adhesion molecule, NF-κB, MAPK

Introduction

Atherosclerosis is one of the most important factors contributing to cardiovascular disease and, therefore, represents a serious threat to public health. Many studies have suggested an important role for vascular smooth muscle cells (VSMCs) in the initiation of atherosclerosis [1]. As the disease progresses, VSMCs are in proximity to and physically interact with inflammatory leukocytes, which perform a very important function in further exacerbating the disease [2]. Atherosclerosis is accompanied by increased levels of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) or intracellular adhesion molecule-1 (ICAM-1) in the development of atherosclerosis and plaque instability [3]. The expression of these molecules was detected on smooth muscle cells of plaques with an increase after vascular injury and could promote leukocyte recruitment to the atherosclerotic vascular wall [4]. In addition, adhesion molecule expression is influenced by the cytokine milieu including TNF-α in vascular cells [5]. Therefore, inhibiting the expression of these adhesion molecules on VSMCs may be an important therapeutic approach for atherosclerosis.

Carvacrol (2-methyl-5-isopropylphenol) is a phenolic monoterpenes abundantly present in the essential oils produced by aromatic plants and spices of lamiaceae family [6]. Several studies have demonstrated that carvacrol presents pharmacological effects of interest, such as analgesic [7], anti-inflammatory [8], antitumor [9] and antioxidant [10]. It was recently reported that carvacrol inhibits migration and proliferation of RASMCs, thereby attenuating vascular neointimal formation [11]. However, the effects of carvacrol on the expression of adhesion molecules in VSMCs have not been ex-

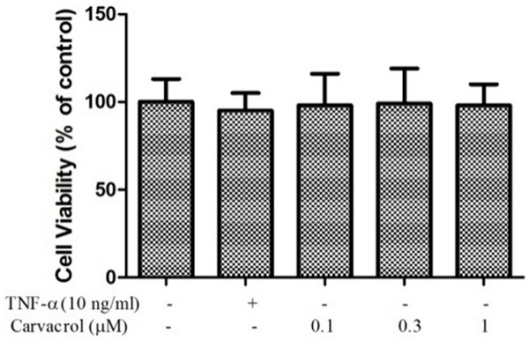

Figure 1. Effect of carvacrol or TNF-α on cell viability. Cells were treated for 24 h with the indicated concentrations of carvacrol or TNF-α. All experiments were repeated at least three times and all data are reported as means ± SD.

plored. Therefore, in this study, we examined the effects and mechanisms of action of carvacrol in the context of adhesion molecule accumulation induced by TNF-α.

Materials and methods

Antibodies and reagents

Carvacrol was purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM medium and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Carlsbad, CA). pGL3-NF-κB, pCMV-β-gal and the luciferase assay system were obtained from Invitrogen (Carlsbad, CA, USA). Antibodies against ICAM-1, VCAM-1, IκB-α, p65 NF-κB, JNK, phospho-JNK (p-JNK), phospho-38, p38, ERK, phopho-ERK (p-ERK) and β-actin were purchased from Abcam (Cambridge, UK). Other chemical reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Cell culture

VSMCs (A10) were purchased from ATCC (Rockville, MD) and were grown in DMEM medium supplemented with 200 mg/ml G418, 100 mg/ml streptomycin, 100 IU/ml penicillin, and 10% heat-inactivated FBS in a humidified atmosphere containing 5% CO_2 incubator at 37°C. For subculturing, the cells were detached using 0.125% trypsin containing 0.01 M EDTA. Cells used in this study were from the first to sixth passage. All experiments were carried out with the same batch of A10, which were from single donor [12].

Cell viability assay

Cells were seeded in 96-well plates and treated with carvacrol (0.1, 0.3, 1 μM) or TNF-α (10 ng/ml) for 24 h in the complete medium or serum-free medium. 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT, 1 mg/ml) was added and incubated at 37°C for 1 h. The formazan granules generated by the live cells were dissolved in 100% DMSO, and absorbance at 550 nm was monitored using a multi-scanner enzyme-linked immunosorbent assay auto-reader [13].

qRT-PCR

At the end of the experiment, total RNA was isolated from the cells using the miRNeasy kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was achieved with the High Capacity cDNA Kit according to the manufacturer's instructions. Quantitative PCR (qPCR) reactions were performed in a 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Green Supermix (Invitrogen, Carlsbad, CA). Relative expression levels of genes were calculated using the $2^{-\Delta\Delta CT}$ method and the house keeping gene β-actin was utilized as a control [14, 15]. The primer sequences used for qRT-PCR were as follows: VCAM-1 forward, 5'-CCCAAGGATCCAGAGATTCA-3' and VCAM-1 reverse, 5'-TAAGGTGAGGGTGGCATTTC-3'; ICAM-1 forward, 5'-CCTGTTTCCTGCCTCTGAAG-3' and ICAM-1 reverse, 5'-GTCTGCTGAGACCCCTCTTG-3' [16].

Transfection and reporter assays

VSMCs (5×10^5 cells/ml) were plated into each well of a 6-well plate. The cells were transiently co-transfected with the plasmids, pGL3-NF-κB and pCMV-β-gal using Lipofectamine Plus according to the manufacturer's protocol. Briefly, a transfection mixture containing 0.5 μg pGL3-NF-κB and 0.2 μg pCMV-β-gal was mixed with the Lipofectamine Plus reagent and added to the cells. After 4 h, the cells were pretreated with carvacrol for 2 h followed by the addition of TNF-α for 4 h, and then lysed with 200 μl of lysis buffer (24 mM Tris-HCl (pH 7.8), 2 mM EDTA, 2 mM dithiotreitol, 1% Triton X-100 and 10% glycerol) and 10 μl of cell lysates were used for luciferase activity assay. The luciferase and β-galactosidase activities were determined. The values shown represent an average of three independent transfections, which were normalized with β-galactosidase activity. Each

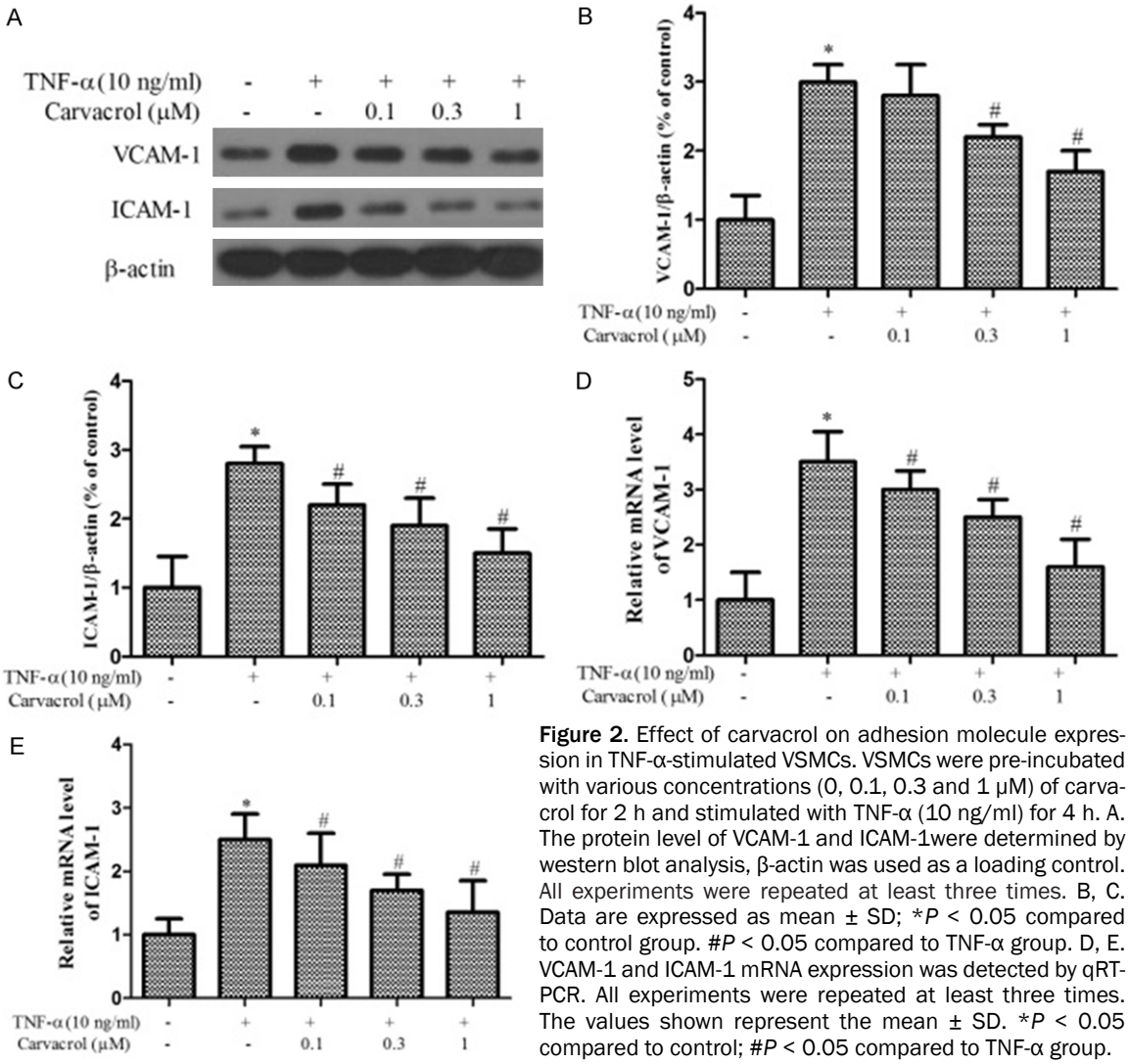

Figure 2. Effect of carvacrol on adhesion molecule expression in TNF-α-stimulated VSMCs. VSMCs were pre-incubated with various concentrations (0, 0.1, 0.3 and 1 μM) of carvacrol for 2 h and stimulated with TNF-α (10 ng/ml) for 4 h. A. The protein level of VCAM-1 and ICAM-1were determined by western blot analysis, β-actin was used as a loading control. All experiments were repeated at least three times. B, C. Data are expressed as mean ± SD; **P* < 0.05 compared to control group. #*P* < 0.05 compared to TNF-α group. D, E. VCAM-1 and ICAM-1 mRNA expression was detected by qRT-PCR. All experiments were repeated at least three times. The values shown represent the mean ± SD. **P* < 0.05 compared to control; #*P* < 0.05 compared to TNF-α group.

transfection was carried out in triplicate and experiments were repeated three times [17].

Western blot

Total cellular proteins were extracted by incubating cells in lysis buffer according to the operating protocols. The protein concentrations in the cell lysates were determined using a bicinchoninic acid assay protein assay kit (Gibco, Rockville, MD). The same amount of protein from each sample was separated by sodium SDS-PAGE on a 12% gel and electrophoretically transferred to a nitrocellulose membrane (Gibco, Rockville, MD). Mouse monoclonal anti-VCAM-1, anti-ICAM-1, IκB-α, p65 NF-κB, JNK, p-JNK, p38, p-p38, ERK and p-ERK were then used as the primary antibody and anti-mouse IgG monoclonal antibody conjugated with horseradish peroxidase was used as the secondary antibody. Protein bands were detected using the West Femto system (Pierce, Rockford, IL). Bands were visualized using ECL and semi-quantitative analysis was performed using β-actin as a protein loading control [18].

ROS production assay

ROS production was determined as previously described [19]. CMH_2DCFDA, a redoxsensitive fluorescent dye, was used to evaluate the intracellular ROS level by flow cytometry. VSMCs (3×10^6 cells/ml) were pretreated with a various concentrations of carvacrol for 2 h, followed by addition of TNF-α (10 ng/ml) for 4 h. The cells were then stained for 15 min at 37°C with 5 μM CMH_2-DCFDA. The cells were kept on ice in the dark and at least 10,000 cells for each sample

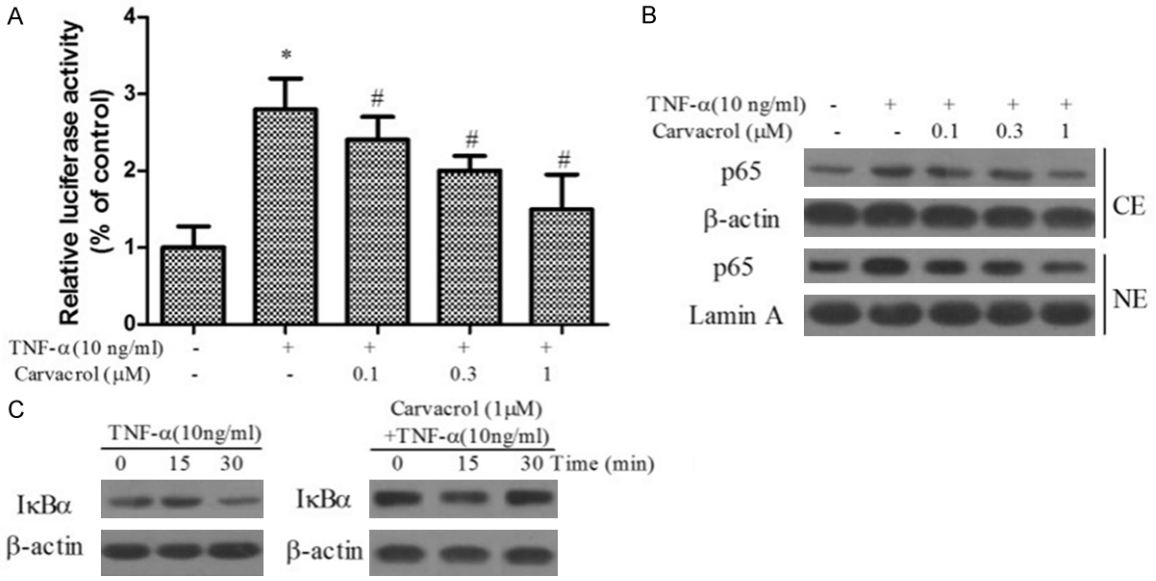

Figure 3. Effects of carvacrol on NF-κB activation and IκBα degradation in TNF-α-stimulated VSMCs. NE, nuclear extracts; CE, cytoplasmic extracts. A. VSMCs were transfected with a pGL3-NF-κB-Luc reporter plasmid and pCMV-β-gal, pretreated with various concentrations of carvacrol for 2 h, and stimulated with TNF-α for 4 h. The values shown represent the mean ± SD. *$P < 0.05$ compared to control group. #$P < 0.05$ compared to TNF-α group. B. VSMCs were pre-incubated with or without various concentrations of carvacrol for 2 h, and then treated with TNF-α for 4 h. The protein level of p65 was detected by Western blotting to analyze the translocation of NF-κB. Lamin A and β-actin were used as loading controls for nuclear and cytosolic protein fractions, respectively. All experiments were repeated at least three times. C. The whole cell lysates of VSMCs were analyzed by Western blotting with anti-IκBα antibody. β-actin was used as a loading control. All experiments were repeated at least three times.

were analyzed using a Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA). The changes in the level of intracellular ROS are expressed as a percentage of cells not treated with TNF-α or carvacrol.

Statistical analysis

Data are reported as mean ± SD. All data analysis was performed with the use of SPSS 19.0 statistical software. ANOVA or Student's t test were used to determine statistical significance with $P < 0.05$.

Results

Effect of carvacrol on VSMCs viability

To examine the effect of carvacrol on cell viability, VSMCs were treated for 24 h with the various concentrations of carvacrol or TNF-α. As shown in **Figure 1**, no significant cytotoxicity of carvacrol or TNF-α was observed. These results indicated that carvacrol or TNF-α was therefore not considered to have any appreciable cytotoxicity in VSMCs.

Effect of carvacrol on adhesion molecule expression in TNF-α-stimulated VSMCs

Expression of the VCAM-1 and ICAM-1 gene has been shown to be increased in early atherosclerosis and within atherosclerotic plaques [17]. Therefore, to investigate the anti-inflammatory effect of carvacrol on VSMCs, we examined the expression levels of adhesion molecules including VCAM-1 and ICAM-1 by Western blot and qRT-PCR. As shown in **Figure 2A-C**, VCAM-1 and ICAM-1 protein expression induced by TNF-α treatment, whereas carvacrol downregulated TNF-α induced VCAM-1 and ICAM-1 protein level in a concentration-dependent manner. Similarly, the level of VCAM-1 and ICAM-1 mRNA induced by TNF-α were significantly decreased in response to carvacrol in a concentration-dependent manner (**Figure 2D** and **2E**).

Effect of carvacrol on TNF-α-induced NF-κB activation in VSMCs

Since it is well established that NF-κB is crucial for the induction of adhesion molecules by TNF-α [20] and carvacrol has an inhibitory ef-

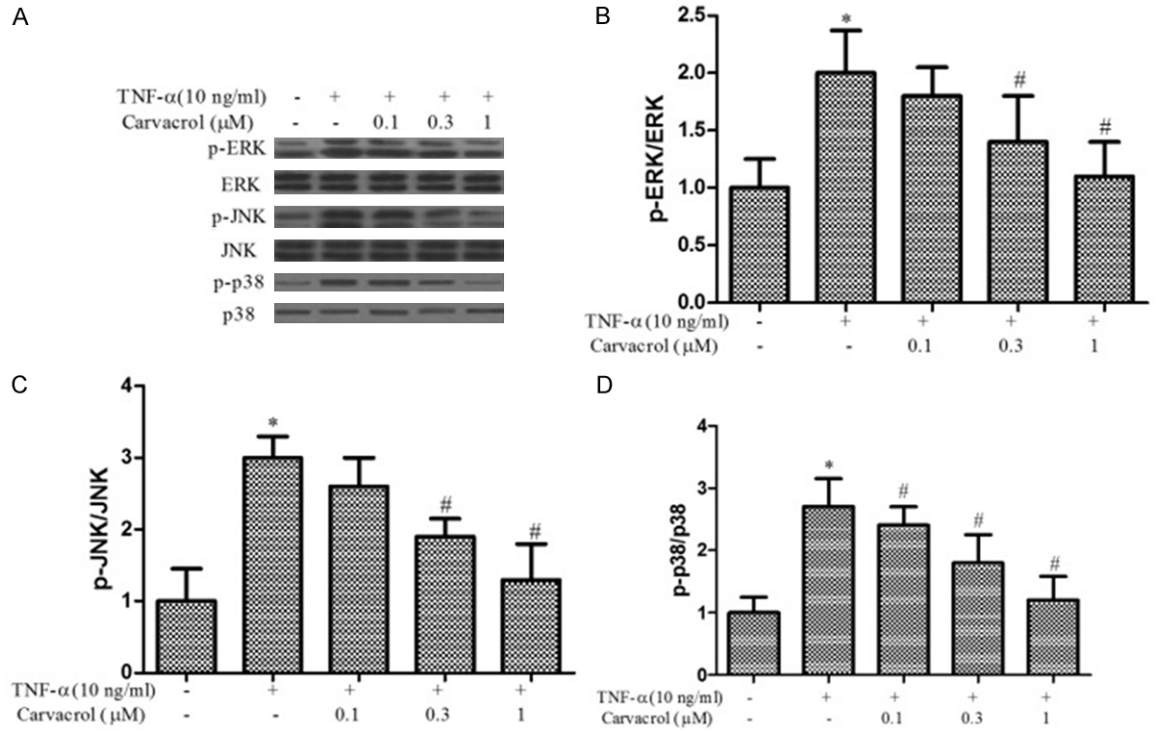

Figure 4. Effect of carvacrol on TNF-α-induced phosphorylation of MAPKs in VSMCs. A. The protein expression levels of ERK, p-ERK, JNK, p-JNK, p38 and p-p38 were determined using western blotting with corresponding antibodies. β-actin was used as an internal control. B-D. Data are expressed as mean ± SD; **P*< 0.05 compared to control group. #*P* < 0.05 compared to TNF-α group.

fect on adhesion molecule expression, we examined whether the inhibitory effect of carvacrol on TNF-α-induced adhesion molecule expression is mediated by NF-κB activation. We used transcriptional activation assays to determine whether carvacrol affects NF-κB dependent transcription. TNF-α treatment resulted in an approximately 2.8-fold increase in luciferase activity, and this increase was significantly inhibited by carvacrol treatment at 1 μM (**Figure 3A**). Since the p65 subunit of NF-κB has been demonstrated to exert critical activity in the transcription of many inflammatory genes [21], we also investigated the effect of carvacrol on the expression of p65 NF-κB protein. As shown in **Figure 2B**, pretreatment of VSMCs with carvacrol reduced p65 NF-κB translocation to the nuclear fraction, suggesting that carvacrol inhibits the TNF-α-induced nuclear translocation of NF-κB.

The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα [22]. Therefore, we examined the effect of carvacrol on TNF-α-induced degradation of IκBα. Treatment of the cells with TNF-α caused IκBα degradation as compared to untreated, whereas in carvacrol-pretreated cells TNF-α prevented the degradation of IκBα.

Effect of carvacrol on TNF-α-induced phosphorylation of MAPKs in VSMCs

It has been reported that the activation of the MAP kinase (MAPK) in response to TNF-α treatment increases the expression of adhesion molecules [23]. Therefore, the p38 MAPK, ERK and JNK kinase pathways were examined to determine if the inhibitory effect of carvacrol on the expression of adhesion molecules is dependent on the MAPK pathways. **Figure 4A-D** shows that TNF-α clearly stimulated an increase in the levels of activation of ERK, JNK and p38 MAPK in untreated cells. However, the induced MAPK activity was significantly inhibited by pretreatment with carvacrol. These results suggest that carvacrol may reduce TNF-α-induced VCAM-1 and ICAM-1 expression by reducing the activation of MAPK pathway.

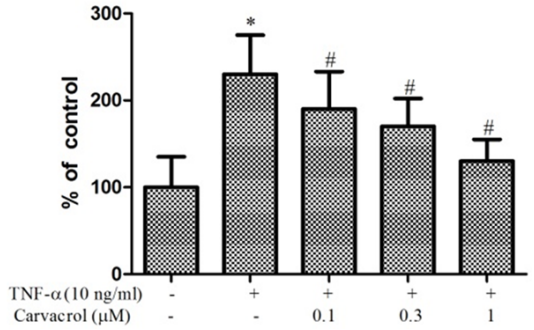

Figure 5. Effects of carvacrol on the production of ROS in TNF-α-stimulated VSMCs. VSMCs were treated with carvacrol at the concentrations indicated for 2 h followed by the stimulation with TNF-α for 4 h. The values shown represent the mean ± SD. *P < 0.05 compared to control group. #P < 0.05 compared to TNF-α group.

Effects of carvacrol on the production of ROS in TNF-α-stimulated VSMCs

It has been suggested that TNF-α induced ROS production activates NF-κB in vascular cells [24]. Therefore, the effect of carvacrol on the production of TNF-α-induced ROS was investigated. Carvacrol significantly suppressed the production of TNF-α-induced ROS in a concentration-dependent manner (**Figure 5**), implicating that carvacrol inhibits NF-κB activation via the suppression of ROS production and has an antioxidant activity.

Discussion

Inflammation is related to the pathogenesis of a variety of diseases including atherosclerosis, multiple sclerosis, bronchitis, gastritis and rheumatoid arthritis [25]. In this study, we were particularly interested in the pathogenesis of atherosclerosis. The expression of adhesion molecules was evoked by inflammatory cytokines such as TNF-α in the atherosclerotic lesion [26]. VSMCs express the cellular adhesion molecules ICAM-1 and VCAM-1 in many inflammatory diseases including atherosclerosis [27]. Thus, modulation of expression of the cell adhesion molecules can be considered as a promising strategy for improving the treatment of atherosclerosis diseases.

Carvacrol has been reported to have a wide range of protective properties against disease states including inflammation, tumor growth, oxidative stress, and microbial infection [28]. It was recently reported that carvacrol affects the migration and proliferation of rat aortic smooth muscle cells and on vascular neointima formation [29]. Therefore, the aim in the present study is to investigate the protective effect of carvacrol in the development of atherosclerosis. However, the underlying signaling pathway involved in this process remains largely unclear.

In this study, we evaluated the effects of carvacrol on expression of the muscle adhesion molecules VCAM-1 and ICAM-1 in TNF-α-stimulated VSMCs and its potential mechanism. Carvacrol prevented induction of expression of adhesion molecules in a concentration-dependent manner after stimulation with the TNF-α at the level of protein and mRNA.

NF-κB is well known to regulate downstream genes in inflammatory response related diseases [30]. It has been also shown that activation of transcription factor NF-κB by TNF-α is required for the transcriptional activation of muscle cell adhesion molecules [31]. NF-κB activation was associated with the phosphorylation and degradation of IκB-α and the nuclear translocation of p65 [32, 33]. The present data show that carvacrol inhibited TNF-α-induced NF-κB activation through inhibition of NF-κB p65 and IκBα degradation, suggesting that carvacrol inhibits TNF-α-induced adhesion molecule expression at least partially through inhibition of NF-κB activation.

Multiple MAP kinases play an important role in the signal transduction pathways that regulate cell adhesion molecules expressed on cells in response to external stimuli including TNF-α [34]. There is much evidence that cells may utilize different MAPK signaling pathways for TNF-α-induced expression of adhesion molecules and that the MAPK signaling pathways are differently involved in response to anti-inflammatory compounds. Our data demonstrate that carvacrol significantly inhibited phosphorylation of p38 MAPK, JNK and ERK phosphorylation in TNF-α-stimulated VSMCs. Thus, these results suggest that carvacrol treatment inhibits TNF-α-induced adhesion molecule expression through inhibition of MAPK signaling pathways.

It has been suggested that many of the effects of cytokines on vascular cells could involve increases in ROS [35]. In the present study, pretreatment with carvacrol significantly inhibited the TNF-α induced ROS production in VSMCs,

indicating that carvacrol has an anti-oxidant activity. A previous study showed that ROS activates various transcription factors in cultured vascular cells and may function as a signaling molecule in various pathways leading to MAPK, Akt, NF-κB activation [36]. Therefore, it is possible that the inhibitory effect of carvacrol on the TNF-α induced expression of adhesion molecules and activation of NF-κB, Akt and MAPK is due to its anti-oxidative properties.

In summary, the results of the present study demonstrate that carvacrol prevents the expression of VCAM-1 and ICAM-1 in TNF-α-stimulated VSMCs. These results suggest a pharmacological activity of carvacrol in VSMCs. The action of carvacrol is resulting from the suppression of the NF-κB, MAPK signaling pathways and intracellular ROS production. These data might account, at least in part, for the anti-inflammatory activities of carvacrol. Thus, carvacrol is proposed as an effective new anti-inflammatory agent that may have a potential therapeutic use for preventing the advancement of atherosclerotic lesions.

Disclosure of conflict of interest

None.

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