Possible Mechanism of the Anti-inflammatory Activity of Ruscogenin: Role of Intercellular Adhesion Molecule-1 and Nuclear Factor-κB

Ya-Lin Huang¹,²,†, Jun-Ping Kou¹,†, Li Ma³, Jia-Xi Song¹, and Bo-Yang Yu¹,³,*

¹Department of Complex Prescription of TCM, China Pharmaceutical University, Nanjing 210038, China
²Nanjing Forest-police College, Nanjing 210046, China
³Key Laboratory for Modern Traditional Chinese Medicine, Ministry of Education, Nanjing 210038, China

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Abstract. Ruscogenin (RUS), first isolated from Ruscus aculeatus, also a major steroidal sapogenin of traditional Chinese herb Radix Ophiopogon japonicus, has been found to exert significant anti-inflammatory and anti-thrombotic activities. Our previous studies suggested that ruscogenin remarkably inhibited adhesion of leukocytes to a human umbilical vein endothelial cell line (ECV304) injured by tumor necrosis factor-α (TNF-α) in a concentration-dependent manner. Yet the underlying mechanisms remain unclear. In this study, the in vivo effects of ruscogenin on leukocyte migration and celiac prostaglandin E₂ (PGE₂) level induced by zymosan A were studied in mice. Furthermore, the effects of ruscogenin on TNF-α–induced intercellular adhesion molecule-1 (ICAM-1) expression and nuclear factor-κB (NF-κB) activation were also investigated under consideration of their key roles in leukocyte recruitment. The results showed that ruscogenin significantly suppressed zymosan A–evoked peritoneal total leukocyte migration in mice in a dose-dependent manner, while it had no obvious effect on PGE₂ content in peritoneal exudant. Ruscogenin also inhibited TNF-α–induced over expression of ICAM-1 both at the mRNA and protein levels and suppressed NF-κB activation considerably by decreasing NF-κB p65 translocation and DNA binding activity. These findings provide some new insights that may explain the possible molecular mechanism of ruscogenin and Radix Ophiopogon japonicus for the inhibition of endothelial responses to cytokines during inflammatory and vascular disorders.

Keywords: ruscogenin, endothelial cell, adhesion, intercellular adhesion molecule-1 (ICAM-1), nuclear factor-κB (NF-κB)

Introduction

Ruscogenin (Fig. 1), first isolated from Ruscus aculeatus, has been reported to exert significant anti-inflammatory activities. It acts as an anti-elastase, decreases capillary permeability, and is widely used to treat chronic venous insufficiency and vasculitis in Europe for more than 40 years (1 – 3). In addition, ruscogenin is also a major steroidal sapogenin of Radix Ophiopogon japonicus, a Chinese herb that has been used to treat acute and chronic inflammatory and cardio-vascular diseases for thousands of years (4). The related pharmacological studies have been confirmed activities of the ethanol extract from Ophiopogon japonicus or ruscogenin glycoside in various aspects such as anti-arrhythmia (5), anti-ischaemia (6), anti-thrombosis (7, 8)

†These two authors contribute equally to this work.
*Corresponding author. boyangyu59@163.com
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Fig. 1. Structure of ruscogenin (RUS).
effects; improving liver injury (9); and inhibiting inflammation (10, 11). Our previous studies also showed that ruscogenin significantly inhibited the adhesion of leukocytes to ECV304 cells activated by tumor necrosis factor-α (TNF-α), while it had no remarkable effect on leukocyte adherence to resting ECV304 cells and cell viability, and also inhibited leukocyte migration in vivo. Meanwhile, it did not down-regulate expression of cyclooxygenase-2 (COX-2) mRNA induced by phorbol ester (PMA) in ECV304 cells (12, 13). However, little is known about the anti-inflammatory mechanisms of ruscogenin and Radix Ophiopogon japonicus in endothelial responses to cytokines during inflammatory and vascular disorders.

On the other hand, the recruitment of immune cells to the inflammatory focus is an important facet of an inflammatory response and represents a multistage process involving leukocyte rolling, adhesion to the endothelium, and migration through the endothelial cell junction into the surrounding tissues. Each stage of the inflammatory response is triggered by the expression of specific adhesion molecules on the surface of vascular endothelium (14). Among various endothelial cell adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) is one of the most important active participants in the recruitment of leukocytes to endothelial and inflammatory lesions, which can be up-regulated by various pro-inflammatory cytokines including TNF-α (15). Blocking or inhibition of expression of ICAM-1 can reduce the capability of leukocytes to adhere to the endothelium and subsequently reduce leukocyte emigration (16–20). Furthermore, mutational analysis has shown that the presence of nuclear factor-κB (NF-κB) binding sites is critical for ICAM-1 gene promoter activity (17, 21, 22).

Thus, to elucidate a possible anti-inflammatory mechanism of ruscogenin, in the present study we investigated its effects at several doses on leukocyte emigration and celiac prostaglandin E₂ (PGE₂) content by using peritonitis models and then examined the NF-κB pathway associated with expression of ICAM-1, compared with dexamethasone as a positive drug, whose chemical structure is partially similar.

Materials and Methods

Isolation of ruscogenin
Ruscogenin was isolated from the tubers of Ophiopogon japonicus by successive chromatographic steps and the purity analyzed by high performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD) (23) was 98.6%. The structure was confirmed by comparing its spectrum data (¹H, ¹³C nuclear magnetic resonance, mass spectrometry, infrared spectrum) with those reported in the literature (24).

Animals
Male ICR mice weighing 25–30 g were obtained from the Experimental Animal Center of China Pharmaceutical University (Nanjing, China). They were kept in plastic cages at 22 ± 2°C with free access to pellet food and water and on a 12-h light/dark cycle. This study complied with current ethical regulations on animal research (National Research Council of USA, 1996) and related rules of our university. All animals used in the experiment received humane care.

Zymosan A–induced peritonitis in mice
Zymosan peritonitis was induced as we previously reported (12). Briefly, mice were administered orally with ruscogenin at doses of 0.3, 1, and 3 mg/kg and dexamethasone at 3 mg/kg; and then they were injected intraperitoneally with 1 mg zymosan A in 0.5 ml of sterile phosphate-buffered saline (PBS) 1 h later. After 4 hours, animals were killed with decapitation, and peritoneal cavities were lavaged with 5 ml of ice-cold PBS containing 3 mM ethylenediaminetetraacetic acid (EDTA). Aliquots of the lavage fluid were then stained with Turk’s solution, and total cell counts were performed using a hemocytometer under a light microscope. The remaining fluid was centrifuged. The supernatant was diluted (1:10) with saline for further detection of the content of PGE₂, considering the participation of prostanoids in zymosan-evoked innate immune responses. The absorbance at 278 nm was determined by a DU640 Nucleic acid and Protein analyzer (Beckman, Fullerton, CA, USA) to indicate the content of PGE₂ (25).

Cells and cell culture
ECV304 cells, which phenotypically show many endothelial features, particularly for ICAM-1 expression, were obtained from Shanghai cell collection of the Chinese Academy of Science. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated newborn calf serum (NCS), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere.

RNA extraction and semi-quantitative reverse-transcription-PCR (RT-PCR)
RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer. Approximately 1 μg of the total RNA was added to each cDNA synthesis reaction using the RT preamplification system (Promega, Madison, WI, USA).
The reaction was carried out for 1.5 h at 37°C. Following cDNA synthesis, amplification was performed using the following primers (Genebase, Shanghai, China): ICAM-1, 5'-GCAGCGGCGTGCTATAA-3' (sense) and 5'-CCCATAGGCAGCAGT-3' (antisense); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-ATT CAACGGCACAGTCAAGG-3' (sense) and 5'-GCA GAAGGGCGGAGTGA-3' (antisense). PCR cycle conditions were: 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s for 30 cycles. After amplification, PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide dyeing.

Quantitative (real-time) RT-PCR

Quantitative RT-PCR analysis of ICAM-1 transcripts was carried out using cDNA that was prepared exactly as described above. According to the reported method (26) and the kit manual of SYBR® Premix Ex Taq™ (Takara, Dalian, China), the reactions were performed in 25 μl of a SYBR® premixed Ex Taq™ solution containing 1 μl cDNA and 20 μM primers. The primers for ICAM-1 were CCTGATGGGCAGTCAACAGCTA (sense) and 20 μM primers. The primers for ICAM-1 were GCACCGTCAAGGCTGAGAAC (antisense) and 5'-GAAGGGCGGAGTGA-3' (antisense). The PCR protocol for ICAM-1 and GAPDH consisted of 35 cycles of 95°C for 5 s and 60°C for 20 s. All real-time PCR reactions were performed in triplicate. Gene expression levels were normalized by the expression of GAPDH, a non-regulated reference gene.

Flow cytometry

The flow cytometry assay was performed according to the previously reported protocol (27) with some modifications. ECV304 cells were washed twice with cold PBS containing 1% of fetal calf serum (FCS) and 0.1% of NaN₃ and then resuspended in 100 μl of fluorescein isothiocyanate (FITC)-conjugated mouse anti-human ICAM-1 antibody solution (Jingmei Biotech, Shenzhen, China) and incubated away from light. After 30 min, the cells were washed and fixed with paraformaldehyde (0.5%, 0.5 ml). The fluorescence intensity of the cell population was analyzed by flow cytometry on a FACScan (Becton Dickinson, San Jose, CA, USA).

Nuclear extracts

Nuclear extracts were obtained from ECV304 cells according to the reported method (28). The cells were incubated in cell lysis buffer, allowed to swell on ice for 5 min, and then centrifuged at 3,300 × g for 15 min. The nuclear pellet was resuspended in nuclear extraction buffer and incubated for 30 min at 4°C. The extracted nuclei were pelleted at 25,000 × g for 15 min at 4°C and the supernatant was collected as nuclear extract.

Western blotting

Nuclear proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a standard protocol as described (29). Samples were resuspended in a sample buffer and loaded on 12% acrylamide-bisacrylamide gels. After electrophoresis, proteins were transferred to a PVDF membrane. Blots were blocked by 5% nonfat dry milk. Immunoblotting was performed with the designated antibodies (Boster, Wuhan, China), and detection was performed with an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions.

Electrophoretic mobility shift assay (EMSA)

EMSA for DNA-binding activity of NF-κB was carried out with a non-radioactive EMSA kit (Roche, Mannheim, Germany). The nuclear extract and the labeled oligonucleotide (Invitrogen) were incubated in a binding buffer at room temperature for 1 h, and then the mixture was subjected to electrophoresis on a nondenatured polyacrylamide gel (6%), followed by an electrotransfer to a positively charged nylon membrane. The membrane was subsequently treated with antidigoxigenin-Ap Fab fragments (Roche). The chemiluminescent substrate CSPD (Roche) was added and the resulting chemiluminescence was detected by autoradiography. The specificity of the NF-κB–binding complex was tested by adding excess unlabeled oligonucleotide.

Statistical analysis

Results were each expressed as the mean ± S.D. Data were analyzed by a one-way ANOVA, followed by Student’s two-tailed t test for comparison between two groups and Dunnett’s test when the data involved three or more groups. P<0.05 was considered to be significant.

Results

Effects of ruscogenin on zymosan A–induced leukocyte migration and PGE₂ level in mice

Intraperitoneal injection of zymosan A produced significant leukocytes, mainly neutrophil accumulation into mouse peritoneal cavities and invoked PGE₂ release at the same time. Ruscogenin markedly inhibited leukocyte emigration at doses of 1 and 3 mg/kg, and the latter showed similar potency to that of dexamethasone.
Ruscogenin Downregulates ICAM-1 and NF-κB

at 3 mg/kg as a positive control drug (Fig. 2A). However, ruscogenin at the tested doses did not exert any remarkable effects on PGE₂ content in peritoneal fluid, in contrast to a significant efficacy of dexamethasone (Fig. 2B).

**Effect of ruscogenin on TNF-α–induced mRNA expression of ICAM-1 in ECV304 cells**

As shown in Fig. 3 (A and B), ICAM-1 mRNA was strongly up-regulated after TNF-α incubation (compare lanes 1 and 2 of Fig. 3A) and was inhibited by ruscogenin at concentrations of 0.1 and 1 μM in a concentration-dependent manner (compare lanes 2 to 5 of Fig. 3A). The positive drug, dexamethasone also showed significant inhibition at 1 μM (compare lanes 2 and 6 of Fig. 3A). Such results were confirmed by real-time PCR.
(Fig. 3C), which showed that ruscogenin at concentrations of 0.1 and 1 μM or dexamethasone at 1 μM significantly inhibited the increase of ICAM-1 mRNA expression induced by TNF-α.

**Effect of ruscogenin on TNF-α–induced protein expression of ICAM-1**

The ECV304 cells stimulated with TNF-α expressed high protein levels of ICAM-1 when analyzed by flow cytometry. Similar to dexamethasone, ruscogenin decreased the ICAM-1 level in a concentration-dependent manner and produced 61.1% decrease at 1 μM (Fig. 4), which was consistent with our findings at the mRNA level.

**Effect of ruscogenin on TNF-α–induced NF-κB accumulation in the nucleus**

As shown in Fig. 5 (A and B), nuclear accumulation of NF-κB p65 was reinforced by 0.5 h of TNF-α treatment in ECV304 cells. At the same time, pretreatment with ruscogenin or dexamethasone at 1 μM for 1 h before TNF-α stimulation remarkably attenuated increase of NF-κB p65.

**Effect of ruscogenin on TNF-α–induced NF-κB DNA binding activity**

As shown in Fig. 6, there was a low level of NF-κB in the nucleus of unstimulated cells (lane 1). Upon stimulation with TNF-α, there was an increase in levels of NF-κB, thus causing substantial retardation in the mobility of the labeled oligonucleotide (lane 2), with high intensity of the shifted band. The enhanced DNA

![Fig. 4](image1.png)

Fig. 4. Effect of ruscogenin (RUS) on ICAM-1 protein level in ECV304 cells by flow cytometric analysis. Cells were pretreated with RUS (0.01, 0.1, and 1 μM) and dexamethasone (DEX) (1 μM) for 1 h and then stimulated with TNF-α (10 ng/ml) for 4 h. Data are shown as means ± S.D. for three separate experiments. *P<0.05, **P<0.01, significantly different from the control group.

![Fig. 5](image2.png)

Fig. 5. Effect of ruscogenin (RUS) on TNF-α–induced NF-κB p65 accumulation in the nucleus evaluated by Western blotting. The cells pretreated with RUS (1 μM) or dexamethasone (DEX) (1 μM) were incubated with TNF-α (10 ng/ml) for another 0.5 h and then nuclear proteins were extracted. Results were expressed as the ratio of NF-κB p65 protein relative to β-actin protein levels. A: Representative assay of 3 separate Western blotting experiments. B: Quantitative histogram of NF-κB p65 protein expression from three separate experiments. Data are shown as means ± S.D. for three separate experiments. **P<0.01, significantly different from the control group.

![Fig. 6](image3.png)

Fig. 6. Effect of ruscogenin (RUS) on TNF-α–induced DNA binding activity of NF-κB determined by EMSA assay. The cells were pretreated with RUS (0.01, 0.1, and 1 μM) and dexamethasone (DEX) (1 μM) for 1 h and then cultured with TNF-α (10 ng/ml) for 0.5 h before nuclear proteins were extracted. The arrow indicates the NF-κB complex. Free labeled probes are also indicated. The specificity of the NF-κB–binding complex was tested by adding excess unlabeled oligonucleotide (shown as blank control).
binding activity was obviously inhibited by pretreatment of ruscogenin at concentrations of 0.1 and 1 μM or dexamethasone at 1 μM (compare lane 2 with lanes 4 – 6). The specificity of the NF-κB–DNA complex was confirmed by adding excess unlabeled oligonucleotide (lane 7).

**Discussion**

In this paper, we explored the possible anti-inflammatory mechanisms of ruscogenin based on our former studies. The results showed that ruscogenin significantly suppressed zymosan A–evoked peritoneal total leukocyte migration, while had no obvious effect on PGE_2_ level. It also inhibited TNF-α-induced over-expression of ICAM-1 at both the mRNA and protein levels and suppressed NF-κB activation considerably by decreasing NF-κB p65 translocation and DNA binding activity.

It has been reported that pleurisy and peritonitis induced by zymosan have long been used as important models to examine the inflammation by evaluating exudate formation and leukocyte infiltration. Furthermore, significant production of prostanooids in zymosan-induced peritonitis is described, suggesting the participation of prostanooids in zymosan-evoked innate immune responses (30, 31). We observed the activity of ruscogenin at three doses on zymosan–induced leukocyte migration, considering the crucial role of the migration of leukocytes to inflammatory area in the progress of inflammation. The migration of leukocytes to the celiac cavity was significantly enhanced by zymosan A in mice. Against the increase, the treatment with ruscogenin showed a remarkable inhibition (Fig. 2A). However, it had no marked effect on PGE_2_ content in peritoneal fluid, which was different from the positive drug dexamethasone (Fig. 2B). These findings were consistent with our previous results (12) and confirmed that ruscogenin exerted significant anti-inflammatory activity without affecting the cyclooxygenase-2 (COX-2) pathway, which might be connected with inhibition of adhesion molecule expression or reduction of other inflammatory mediator release etc.

On the other hand, it has been widely accepted that vascular endothelial cells play an important and indispensable role in the inflammatory reaction, which provided traffic signals for leukocytes adhesion and transmigration (32, 33). Among them, ICAM-1 is one of the most important endothelial cell surface adhesion molecules (17 – 20). The interactions between ICAM-1 with the β_3integrin counter receptors on leukocytes, in the presence of an activator molecule, could contribute to the arrest of leukocytes along the endothelial cell surface (34). Mice with ICAM-1-deficiency showed a 60% – 70% reduction in acute leukocyte emigration into the peritoneum during peritonitis (35). Thus, we hypothesized that ruscogenin might exert anti-inflammatory and anti-adhesive activities by affecting ICAM-1 expression. Consistent with our assumption, the present data revealed that ruscogenin significantly inhibited the increase of ICAM-1 mRNA expression induced by TNF-α in ECV304 cells (Fig. 3). Meanwhile, ruscogenin suppressed the TNF-α–induced overexpression of ICAM-1 by 61.1% at most in ECV304 cells at the protein level (Fig. 4) without inhibiting the constitutive expression of ICAM-1 (data not shown), indicating that ruscogenin was able to abrogate the TNF-α–induced adhesion molecule expression at both the mRNA and protein levels.

Furthermore, it has been well established that NF-κB, a major transcription factor in the development of atherosclerotic injury and inflammation, mediates cell migration and endothelial cell activation (28, 36). NF-κB is initially located in the cytoplasm as an inactive form complexed with IκB, an inhibitory factor of NF-κB. Various inducers such as TNF-α cause the release of NF-κB from the complex and its translocation to the nucleus. Then NF-κB interacts with its DNA recognition sites to induce genes, which are responsible for an increased transcription of many cytokines, chemokines, and adhesion molecules including ICAM-1 (17, 22, 37). Whether ruscogenin exerts the inhibitory effects on TNF-α–induced overexpression of ICAM-1 through the NF-κB pathway is worthy of further study. Usually, the activation of NF-κB was characterized by the reinforcement of nuclear accumulation and DNA binding activity of NF-κB. Thus, we next examined the effect of ruscogenin on the activation of NF-κB induced by TNF-α by Western blotting and EMSA. As a result, we observed a remarkable increase in the NF-κB p65 translocation from cytoplasm to nucleus in ECV304 cells stimulated by TNF-α, but the increase was suppressed when ruscogenin (1 μM)-pretreated cells (Fig. 5). The results of EMSA also showed that ruscogenin was able to inhibit the TNF-α–induced enhancement of NF-κB DNA binding capacity (Fig. 6), which suggested that ruscogenin might suppress the TNF-α–induced overexpression of ICAM-1 through the NF-κB pathway.

Structural similarity of drugs usually leads to the similarity of the actions. The anti-inflammatory effect of dexamethasone relies on direct or indirect effects on gene expression by the binding of glucocorticoid receptors to glucocorticoid-responsive elements or other transcription factors (38). Interestingly, possessing partly
similar structures, ruscogenin and dexamethasone acted analogously in many anti-inflammatory mechanisms such as in the inhibition of leukocyte adhesion (13) and migration (Fig. 2A), ICAM-1 expression (Figs. 3 and 4), and NF-κB activation (Figs. 5 and 6). Thus, we hypothesized that ruscogenin might also exert anti-inflammatory and anti-adhesive activities linked with glucocorticoid receptors or a similar signal transduction pathway. However, they acted differently in some situations. As described, ruscogenin had no obvious effect on the increase of PGE₂ in peritoneal fluid of mice (Fig. 2B) and the expression of cyclooxygenase-2 (COX-2) in ECV304 cells (12), suggesting that some structural differences between ruscogenin and dexamethasone resulted in the different protein targets or signaling pathways. Further studies are required to clarify these differences. Investigation of the spectrum of intracellular targets of ruscogenin by affinity chromatography is underway.

In summary, our findings indicate that ruscogenin inhibited the cytokine-induced enhancement of leukocyte adhesion and migration, which is related to suppression of ICAM-1 expression in endothelial cells through inhibition of the NF-κB pathway. Such results reveal some new insights on the molecular mechanisms of its significant anti-inflammatory or anti-thrombotic activities. Additional studies should be undertaken to further elucidate its exact mechanisms such as exploring its direct binding protein target and so on.

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References

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