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Short Communication

Ruscogenin Mainly Inhibits Nuclear Factor-κB but Not Akt and Mitogen-Activated Protein Kinase Signaling Pathways in Human Umbilical Vein Endothelial Cells

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Abstract. Our previous results suggested that ruscogenin inhibited tumor necrosis factor α (TNF-α)–induced leukocyte adhesion, which correlated with its suppression of intercellular adhesion molecule-1 (ICAM-1) expression in endothelial cells. In the present studies, we further examined its effects on the main signaling pathways involved in upregulation of ICAM-1 induced by TNF-α in human umbilical vein endothelial cells (HUVECs). The results showed that ruscogenin significantly suppressed p65 phosphorylation, IκB-α phosphorylation and degradation, and inhibited IκB kinase α (IKKα) and IKKβ activation induced by TNF-α. However, it exerted weak effects on TNF-α–induced phosphorylations of p38, JNK, ERK1/2, and Akt. Overall, our results indicated that downregulation of ICAM-1 expression by ruscogenin in HUVECs might be mediated by nuclear factor-κB (NF-κB), but not by mitogen-activated protein kinase (MAPK) and Akt signaling pathways.

Keywords: ruscogenin, nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK)

Ruscogenin, first isolated from *Ruscus aculeatus*, is also a major steroidal sapogenin of the traditional Chinese herb *Radix Ophiopogon japonicus*, which has been clinically used for a long time to treat acute and chronic inflammatory and cardiovascular diseases. Ruscogenin has been previously found to exert significant anti-inflammatory and anti-thrombotic activities (1), and its possible mechanism could be attributed to its inhibition of intercellular adhesion molecule-1 (ICAM-1) upregulation via nuclear factor-κB (NF-κB) p65 translocation induced by tumor necrosis factor α (TNF-α) (2). However, its intracellular signal transduction pathways remain to be elucidated.

ICAM-1 is an inducible cell surface glycoprotein in endothelial cells, which mediates firm adhesion and diapedesis, and plays an important role in atherosclerosis (3). It has been shown that TNF-α induces ICAM-1 expression, possibly through mitogen-activated protein kinases (MAPKs) (ERK1/2, JNK, and p38), and nuclear factor-κB (NF-κB) signaling pathways (4). The MAPKs affect a variety of intracellular responses, with well-recognized roles in inflammation, cell-cycle regulation, cell death, development, differentiation, senescence, tumorigenesis, and so on. NF-κB plays a pivotal role in chronic and acute inflammatory diseases, autoimmune diseases, and different types of cancer, whose related signaling pathways have become a focal point for intense drug discovery and development efforts (5). The present studies were thus designed to further elucidate the possible signaling pathways of ruscogenin to downregulate ICAM-1 expression in human umbilical vein endothelial cells (HUVECs).

Endothelial cells were isolated from human umbilical cord veins using a previously reported method with several modifications (6). The cells were cultured in Medium 199 (Gibco, Grand Island, NY, USA), which contained 25 μg/ml endothelial cell growth supplement (Sigma, St. Louis, MO, USA), 20% newborn calf serum (PAA, Linz, Austria), 20 units/ml heparin, 100 U/ml

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penicillin, and 100 μg/ml streptomycin, and plated on culture dishes under 5% CO_2 at 37°C. All the cells used in the experiments from passages 2 to 5 were starved in serum-free Medium 199 for 2 h before the experiments. 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 was 98.6% as previously reported (2).

Results were 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 for three or more groups. $P < 0.05$ was considered significant.

Initially, given that ruscogenin caused no obvious inhibition on the basal expression of ICAM-1 in ECV304 cells (2), we verified its inhibitory effect on TNF-α–induced ICAM-1 expression in HUVECs by Western blotting and flow cytometry, and 1 μM of ruscogenin caused about 53.7% inhibition (data not shown), which was similar to our previous findings (2) . Then we investigated its effect on TNF-α–induced p65 (Ser536) phosphorylation in HUVECs. As reported in the literature (7), p65 phosphorylation was induced by TNF-α after stimulation for 5, 15, and 30 min, and pretreatment with ruscogenin at a concentration of 1 μM decreased p65 phosphorylation by about 40% at 5 min and 70% at 15 min (the value of TNF-α treatment alone at each time point subtracted by that of the non-treated control was taken as 100%) (Fig. 1A). In addition, TNF-α–induced p65 phosphorylation was concentration-dependently suppressed by ruscogenin at the concentration of 0.01, 0.1, and 1 μM (Fig. 1B).

Considering that phosphorylation of IκB kinase α (IKKα) and IKKβ and the subsequent activation and degradation of IκB-α are important early steps in NF-κB activation, we then examined the effects of ruscogenin on TNF-α–induced IKKα/β, IκB-α activation, and IκB-α degradation. As shown in Fig. 1C, the degradation of IκB-α occurred as early as 5 min after TNF-α treatment and was maintained for at least 30 min as well as IκB-α phosphorylation (8, 9). The cells pretreated with ruscogenin (1 μM) inhibited TNF-α–induced IκB-α phosphorylation and degradation from 5 to 30 min. Furthermore, similar to previous reports (8 – 10), IKKα and IKKβ were activated by TNF-α treatment within 5 min and phosphorylations were maintained for 15 min, then decreased to a nearly basal level by 30 min, while ruscogenin significantly decreased TNF-α–induced IKKα phosphorylation by about 48.0% at 5 min and 58.9% at 15 min and decreased IKKβ phosphorylation by about 67.8% at 5 min and 91.1% at 15 min (the value of TNF-α treatment alone at each time point subtracted by that of non-treatment control was taken as 100%) (Fig. 1D). Meanwhile, ruscogenin (1 μM) had no significant effects on the basal phosphorylations of p65, IκB-α, and IKKα/β (data not shown).

Akt has been shown to regulate IKK activity and is also involved in the activation of NF-κB by TNF-α (11). Therefore, the effect of ruscogenin on TNF-α–induced Akt activation in HUVECs was then examined. Confirming another group's findings (8), TNF-α (10 ng/ml) induced dephosphorylation of Akt rapidly and transiently at 5 min, with the increase in Akt phosphorylation occurring at 30 min. However, Akt dephosphorylation and phosphorylation were not influenced by ruscogenin (Fig. 2A).

To determine whether ruscogenin affects TNF-α–induced MAPKs activation, we also observed the effects of ruscogenin on TNF-α–induced p38, JNK, and ERK1/2 activations in HUVECs. As reported (12), p38 was activated in 5 min and then was maintained for at least 30 min, ERK1/2 phosphorylation peaked at 15 min and decreased sharply at about 30 min, and p-JNK was gradually increased from 5 to 30 min (Fig. 2: B – D). No significant effect on ERK1/2 activation ($P > 0.05$) was observed with ruscogenin at a concentration of 1 μM. Although p38 activation was just decreased by about 23.9% at 15 min and JNK activation was decreased by about 14.9% at 5 min with ruscogenin, the inhibition effects were so weak, suggesting that ruscogenin does not suppresses ICAM-1 expression through the MAPK signaling pathways.

The present work was designed to further investigate the major intracellular signal transduction pathways involved in the downregulation of ICAM-1 expression by ruscogenin in HUVECs based on our previous work.

It has been reported that TNF-α activation of the ICAM-1 promoter in HUVECs may critically depend on p65 binding to a variant κB site (13). Transcriptional activity of NF-κB/p65 can be regulated by post-translational modifications, such as phosphorylation, that enhance the transcription of target genes in many cases (14). So, we investigated the effect of ruscogenin on TNF-α–induced p65 activation and found that ruscogenin could inhibit phosphorylation of p65 at Ser536 significantly in a dose- and time-dependent manner. It has been reported that IKKα and IKKβ, which constituted the IKK complex with the regulatory subunit NEMO/IKKγ, were responsible for phosphorylation of p65 at Ser536. Furthermore, NF-κB activation is typically mediated by proteasomal degradation of the prototypical and most extensively studied IκB member, IκB-α. This so-called canonical NF-κB pathway is induced by various stimulators, including TNF-α, which triggers signal transduction events that lead to the activation of the IKK complex.

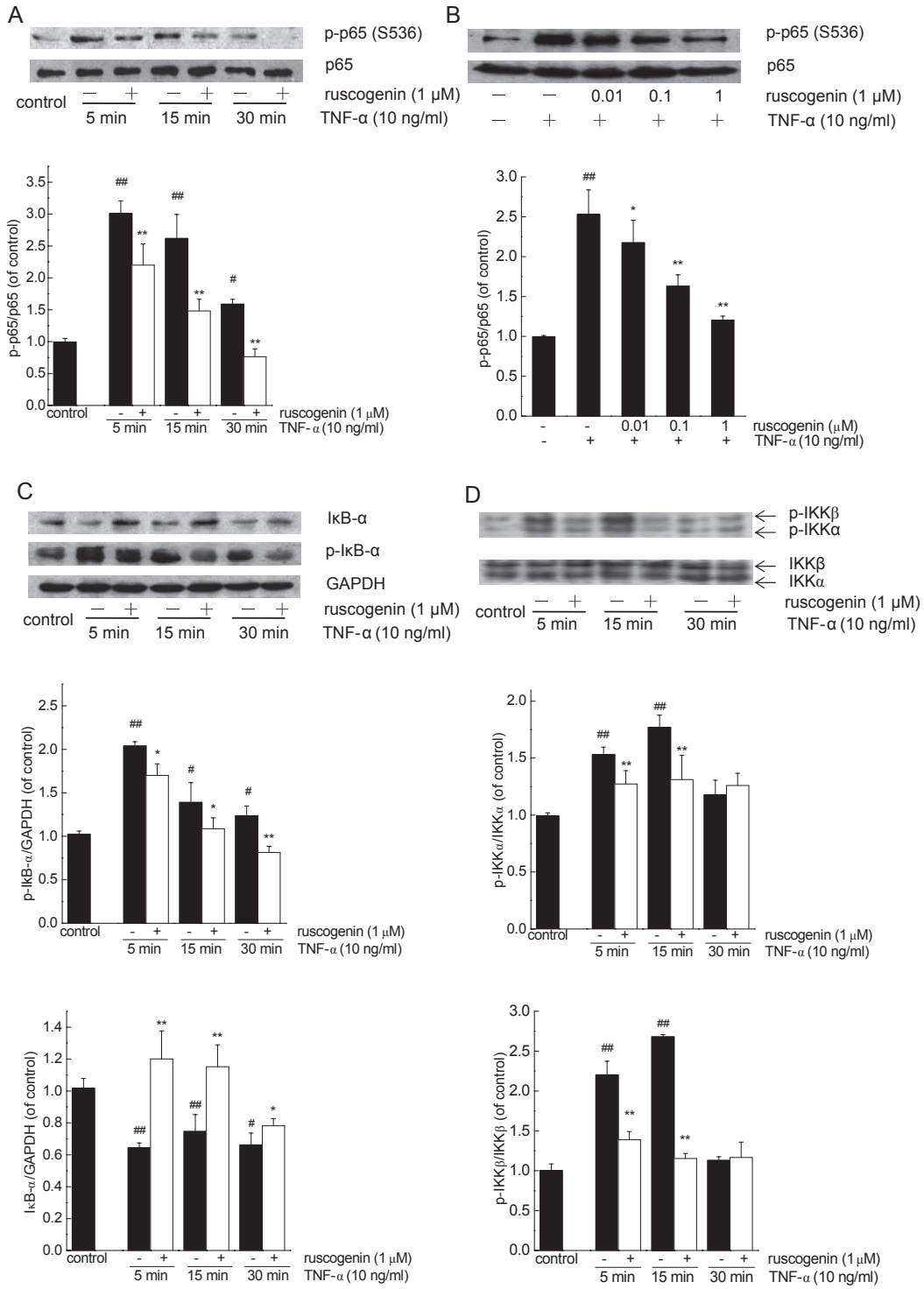

Fig. 1. Effects of ruscogenin on TNF-*α*–induced p65 activation, I*κ*B-*α* degradation, phosphorylation, and IKK*α*/*β* phosphorylation. A, C and D: HUVECs were pretreated with ruscogenin (1 *μ*M) for 1 h before TNF-*α* (10 ng/ml) treatment for 5, 15, and 30 min. B: HUVECs were pretreated with ruscogenin (0.01, 0.1, and 1 *μ*M) for 1 h before TNF-*α* (10 ng/ml) treatment for 15 min. After incubation, p-p65 (Ser536), p-I*κ*B-*α* (Ser32/36), I*κ*B-*α* (Kangchen, Shanghai, China), and p-IKK*α* (Ser180) / *β* (Ser181) (Cell Signaling Technology, Beverly, MA, USA) was determined by Western blotting analysis. The samples were separated by SDS-PAGE (12% gels) and then transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) and detected with the ECL Plus System (Beyotime Biotechnology, Haimen, China) according to manufacturer's instructions (upper panel). The band intensities were assessed by scanning densitometry (lower panel). Data are shown as the means ± S.D. from three separate experiments. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. untreated control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. TNF-*α*–stimulated group without ruscogenin at the indicated time.

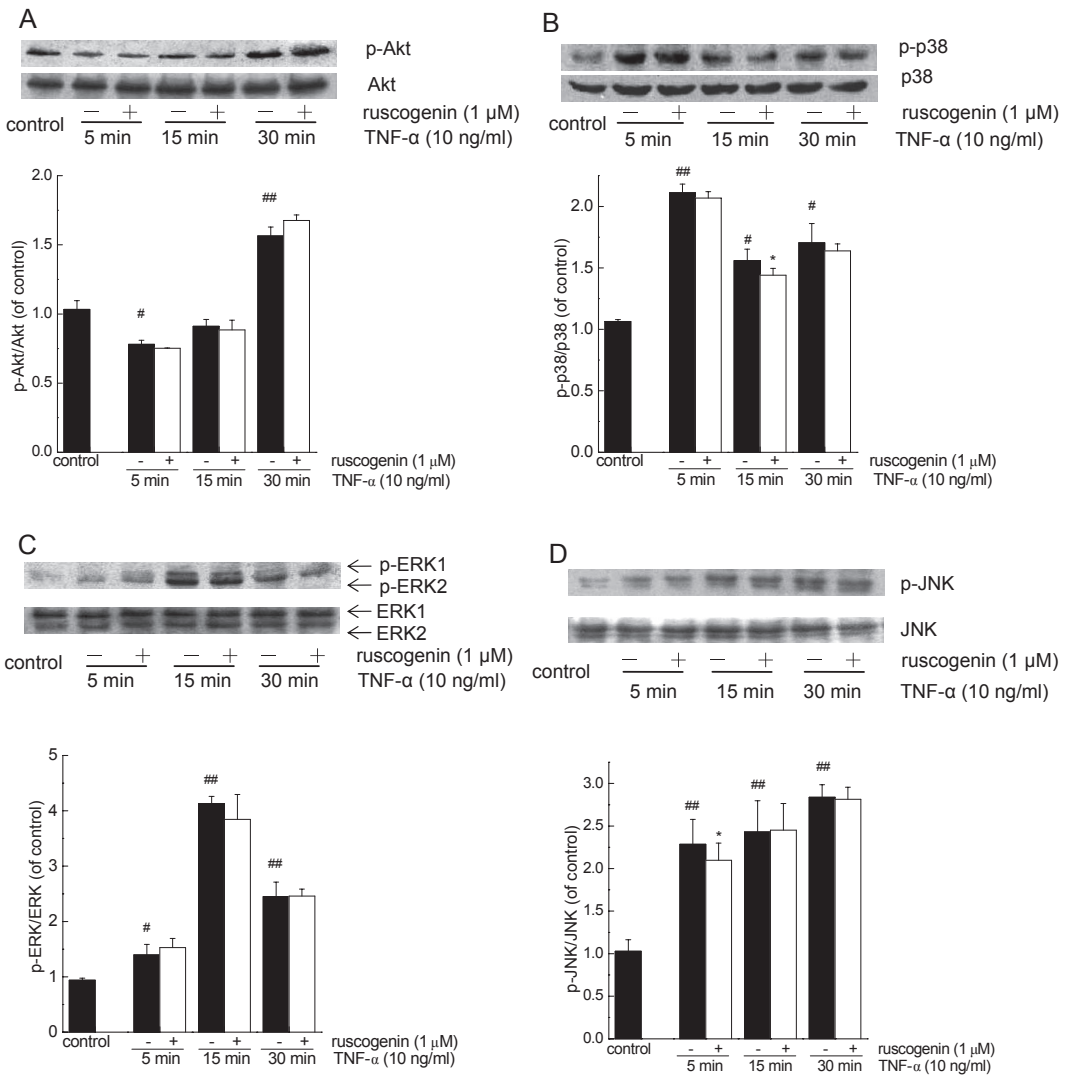

Fig. 2. Effects of ruscogenin on TNF-α–induced Akt, p38, JNK, and ERK1/2 activation. A – D: HUVECs were pretreated with ruscogenin (1 μM) for 1 h before TNF-α (10 ng/ml) treatment for 5, 15, and 30 min; then p-Akt (Ser473), p-p38 (Thr180/Tyr182), p-ERK1/2 (Thr202/Tyr204), and p-JNK (Thr183/Tyr185) (Bioworld, Atlanta, GA, USA) were determined by Western blotting analysis (upper panel), and quantitation of protein phosphorylation was performed by densitometric analysis (lower panel). Data are shown as means ± S.D. from three separate experiments. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. untreated control group. $^{*}P < 0.05$ vs. TNF-α–stimulated group without ruscogenin at the indicated time.

The activated IKK complex phosphorylates IκB-α, predominantly via the action of IKKβ, triggering its proteasomal degradation (14). Consistent with former reports, remarkable phosphorylation and degradation of IκB-α and activation of IKKα/β were induced by TNF-α in our experiments, but the activations were suppressed when cells were pretreated with ruscogenin (1 μM). Interestingly, ruscogenin produced higher inhibition of IKKβ than IKKα, which may be partly explained by the predominant role of IKKβ in the phosphorylation of p65 and IκB-α (15). It is well known that IKKα and IKKβ have been pursued by many groups as targets for the development of therapeutic agents to treat cancers, as well as inflammatory and metabolic diseases (5). Thus, ruscogenin may be a promising leading compound or a new drug candidate for treating the above-related disorders.

On the other hand, Akt has been implicated in the phosphorylation of p65 (11), and NF-κB activity could be modulated through the phosphorylation caused by MAPKs (16). However, our results suggested that ruscogenin did not affect Akt activation and showed weak effects on the activations of p38, JNK, and ERK1/2.

In particular, research has shown that TNF-α signaling occurs through tumor necrosis factor receptor 1 (TNFR1)

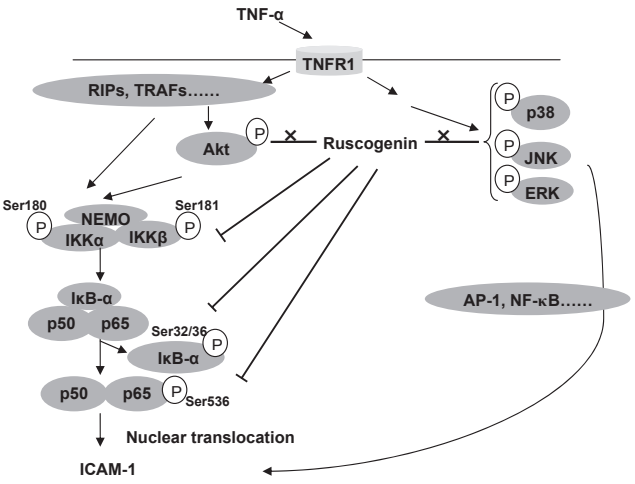

Fig. 3. Schematic diagram of suppression of ICAM-1 expression by ruscogenin in TNF-*α*–stimulated HUVECs mainly through inhibition NF-*κ*B, but not p38, JNK, and ERK signaling pathways.

and/or TNFR2, and TNF-*α*–stimulated ICAM-1 expression on human endothelial cells is mediated exclusively by the NF-*κ*B signaling pathway via the TNFR1 subtype (12). Our results demonstrated that ruscogenin could significantly inhibit TNF-*α*–induced IKK*α*/*β* phosphorylation, thereby impairing the phosphorylation of I*κ*B-*α* and subsequent phosphorylation of NF-*κ*B/p65 at Ser536, without obvious effects on activations of p38, JNK, and ERK1/2, which was a probability as reflected by the responses of TNFR1. There might be upstream potential targets of ruscogenin for regulating IKK, except for Akt, such as TNFR1, receptor-interacting proteins (RIPs), TNFR-associated factors (TRAFs), protein kinase C, glycogen synthase kinase 3*β* (14), and so on (Fig. 3). We also presumed that ruscogenin might have a distinct protein target, such as its binding protein, which could regulate the NF-*κ*B signaling pathway.

In conclusion, ruscogenin suppresses ICAM-1 expression in TNF-*α*–stimulated HUVECs, mainly through inhibition of IKK-p65/I*κ*B-*α* activation, which puts new insights on the molecular mechanism responsible for its anti-inflammatory and anti-thrombotic activities. It would be a valuable small-molecular probe from natural products to explore the new functions of some specific proteins involved in the process of inflammation and thrombosis, especially the NF-*κ*B pathway in the future.

Acknowledgments

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