

Original Research Article

Ruscogenin alleviates palmitic acid-induced endothelial cell inflammation by suppressing TXNIP/NLRP3 pathway

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Abstract

Purpose: To investigate the involvement of ruscogenin in palmitic acid (PA)-induced endothelial cell inflammation.

Method: Cultured human umbilical vein endothelial cells (HUVECs) were divided into five groups: control (normal untreated cells), PA (cell treated with palmitic acid), and PA + ruscogenin (1, 10, or 30 μ M). Cell viability and apoptosis rate were determined using MTT (3-(4,5)-dimethylthiazolyl-2,5-diphenyltetrazolium bromide) and flow cytometry assays, respectively. The levels of cytokines, including interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemo-attractant protein-1 (MCP-1) were determined by an enzyme-linked immunosorbent assay. Western blotting and real-time polymerase chain reaction (RT-PCR) were used to evaluate the underlying mechanisms of action.

Results: PA treatment decreased the viability of HUVECs and induced apoptosis ($p < 0.05$). Ruscogenin attenuated PA-induced cell death in a dose-dependent manner ($p < 0.05$). On the other hand, PA induced an increase in IL-1 β , TNF- α , ICAM-1, MCP-1, TXNIP (thioredoxin-interacting protein), as well as NLRP3 (nucleotide oligomerization domain-, leucine-rich repeat- and pyrin domain-containing protein 3), all of which were attenuated by ruscogenin ($p < 0.05$).

Conclusion: Ruscogenin alleviates PA-induced endothelial cell inflammation via TXNIP/NLRP3 pathway, thereby providing an insight into new therapeutic strategies to treat cardiovascular diseases.

Keywords: Ruscogenin, Palmitic acid, Endothelial cells, Inflammation, TXNIP, NLRP3, Cardiovascular diseases

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INTRODUCTION

Cardiovascular diseases are the leading cause of death worldwide and involve abnormal endothelial cell inflammation [1]. Inflammation leads to abnormal production of reactive oxygen species (ROS), decreased nitric oxide (NO) levels, and endothelial dysfunction and injury,

which also contribute to atherosclerosis [2]. Therefore, inhibiting endothelial inflammation is of great interest to prevent cardiovascular diseases.

Elevation of free fatty acids (FFAs) could induce inflammatory responses and impair vascular reactivity, a known pathogenetic mechanism of

cardiovascular diseases [3]. PA (palmitic acid) is an abundant FFAs, leading to lipid overload that can induce a harmful inflammatory cascade and plays a key role in several chronic diseases, including atherosclerosis [4]. PA is a ligand of endogenous toll-like receptor-4 (TLR4) and promotes inflammatory cytokine secretion, as well as the production of cytoplasmic messengers including ROS, leading to the development of atherosclerosis [5]. Recently, PA was shown to induce activation of NLRP3 (nucleotide oligomerization domain-, leucine-rich repeat- and pyrin domain-containing protein 3) inflammasomes. This eventually leads to the production of active caspase-1 [6] that enhances secretion of proinflammatory interleukin-1 β (IL-1 β) to exacerbate local inflammation [7]. Therefore, PA could induce endothelial inflammation and lead to cardiovascular disease development.

Ruscogenin exerts anti-inflammatory effect against diabetic nephropathy [8] and has been shown to suppress endothelial inflammatory responses in the setting of vascular disorders [9]. However, the effect of ruscogenin on endothelial inflammation and its potential mechanisms remain elusive. In this study, a PA-induced endothelial cell model was used to determine the protective effect and mechanisms of ruscogenin in endothelial inflammation.

EXPERIMENTAL

Cell culture

Human umbilical vein endothelial cells (HUVECs) were acquired from the Chinese Academy of Sciences (Shanghai, China) and cultured in Ham's F-12K medium (Upstate Biotechnology, Lake Placid, NY, USA) supplemented with 10% fetal bovine serum (Upstate Biotechnology) and maintained in a 37 °C incubator with 5% CO₂.

Cell viability and apoptosis

Cultured HUVECs were seeded and pre-treated with indicated concentrations of ruscogenin (1, 10, or 30 μ M) for 2 hours, followed by exposure to 500 μ M PA for another 24 hours. For determination of cell viability, the cells were incubated with 5 mg/mL MTT (3-(4,5)-dimethylthiazazo(-z-y1)-3,5-di-phenyltetrazolium bromide) for 4 hours. After 10 minutes, the absorbance at 450 nm was determined by a microplate reader (Biotek, Winooski, VT, USA). For determination of apoptosis, the cells were harvested and stained with 5 μ L 100 μ g/mL propidium iodide for 30 minutes. After incubation with fluorescein isothiocyanate-conjugated

annexin V, the cells were analyzed using a flow cytometer (Attune, Life Technologies, Darmstadt, Germany).

Determination of inflammatory cytokines

Culture medium from HUVECs pre-treated with or without indicated concentrations of ruscogenin (1, 10, or 30 μ M) were collected and centrifuged, and the supernatant levels of IL-1 β , tumor necrosis factor- α (TNF- α), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemo-attractant protein-1 (MCP-1) were determined by a commercial enzyme-linked immunosorbent assay kits (Thermo Fisher Scientific, Waltham, MA, USA).

Real-time PCR

RNA was extracted from cultured HUVECs with TRIzol (Thermo Fisher Scientific) and reverse-transcribed into cDNAs. Real-time PCR was performed using TransStart® Top Green qPCR SuperMix (Bio-Rad, Hercules, CA, USA) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a negative control. The primer sequences are shown in Table 1.

Table 1: Primer sequences

Gene	Sequence
NLRP3	Forward: 5'-CTCGCATTGGTTCTGAGCTC-3'
	Reverse: 5'-AGTAAGGCCGGAATTCACA-3'
TXNIP	Forward: 5'-GATACCCAGAAAGCTCCTCC-3'
	Reverse: 5'-ACCTCAGTGTAAGTGGGTGG-3'
GAPDH	Forward: 5'-AGTCAGTCTCTCCTTTCAGG-3'
	Reverse: 5'-TCCACCACCCTGTTGCTGTA-3'

Western blot

Proteins were extracted from HUVECs by radioimmunoprecipitation assay buffer (Thermo Fisher Scientific) and quantified by bicinchoninic protein assay (Thermo Fisher Scientific). Extracted proteins (40 μ g) were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% bovine serum albumin and incubated overnight with the following primary antibodies: thioredoxin-interacting protein (TXNIP), NLRP3 (1:2000, Abcam, Cambridge, UK), and β -actin (1:3000, Abcam). After incubation with horseradish peroxidase-conjugated secondary antibody, bands were exposed using enhanced chemiluminescent

detection reagent (Vazyme Biotechnology, Nanjing, China). The signals were analyzed using Image Lab™ software (Bio-Rad).

Statistical analysis

Data are presented as mean \pm SEM and were analyzed with GraphPad Prism software 5.0 (GraphPad Inc., San Diego, CA, USA). Multiple groups were compared with one-way analysis of variance. Differences were considered significant at $p < 0.05$.

RESULTS

Ruscogenin attenuated the PA-induced decrease in HUVECs viability

The possible protective effect of ruscogenin for cell viability was first evaluated in HUVECs. The chemical structure of ruscogenin is shown in Figure 1 A. The HUVECs viability under PA treatment was lower than in the untreated control cells (Figure 1 B). Pretreatment with ruscogenin dose-dependently attenuated the PA-induced decrease in viability (Figure 1 B), suggesting that ruscogenin protected HUVECs from PA-induced cytotoxicity.

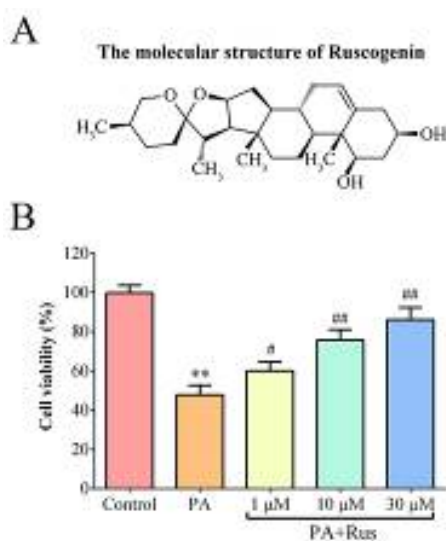


Figure 1: (A) Chemical structure of ruscogenin. (B) Protective effect of ruscogenin on viability in PA-induced HUVECs; ** $p < 0.01$, # $p < 0.05$, ## $p < 0.01$

Ruscogenin blocked PA-induced cell apoptosis

PA promoted HUVECs apoptosis, but this was dose-dependently ameliorated by ruscogenin pretreatment (Figure 2), confirming its protective effect.

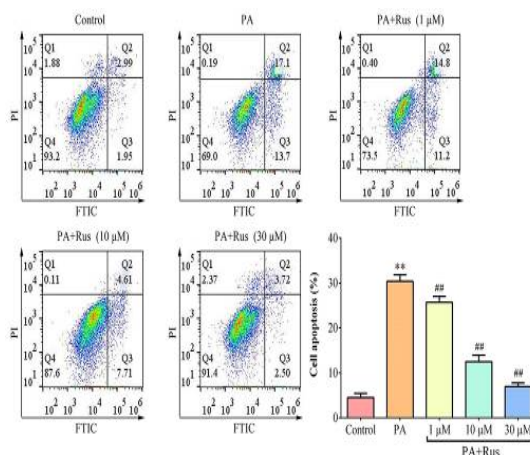


Figure 2: Ruscogenin dose-dependently blocked PA-induced HUVECs apoptosis. ** $p < 0.01$, ## $p < 0.01$

Ruscogenin alleviated PA-induced inflammation in HUVECs

To clarify whether ruscogenin could exert an anti-inflammatory effect on PA-treated HUVECs, inflammatory cytokine secretion was measured. Exposure of HUVECs to PA produced endothelial cell inflammation as evidenced by the increase in IL-1 β , TNF- α , ICAM-1, and MCP-1 (Figure 3). Ruscogenin dose-dependently decreased the levels of IL-1 β , TNF- α , ICAM-1, and MCP-1. These results suggest that ruscogenin effectively alleviated PA-induced inflammation in HUVECs.

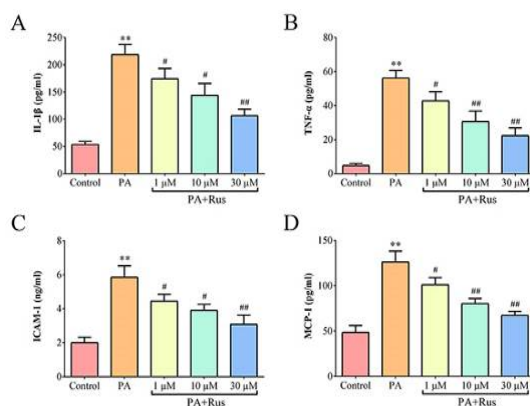


Figure 3: Ruscogenin alleviated PA-induced inflammation in HUVECs. Protective effect of ruscogenin on levels of IL-1 β (A), TNF- α (B), ICAM-1 (C) and MCP-1 (D) in PA-induced HUVECs. ** $p < 0.01$, # $p < 0.05$, ## $p < 0.01$

Ruscogenin inhibited PA-induced activation of TNXIP/NLRP3 signaling

PA-treated HUVECs showed a significant increase in mRNA and protein levels of TNXIP

(Figure 4). However, pretreatment with ruscogenin dose-dependently decreased all four (Figure 4 C). These results indicate that ruscogenin inhibited PA-induced activation of the TNXIP/NLRP3 pathway.

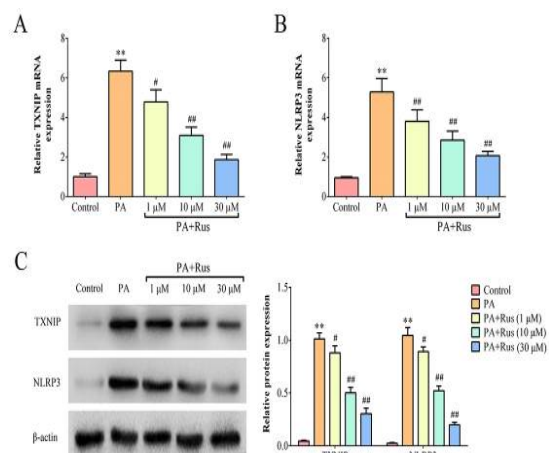


Figure 4: Ruscogenin inhibited PA-induced activation of TNXIP/NLRP3 signaling. (A) Effect of ruscogenin on TNXIP mRNA levels in PA-induced HUVECs. (B) Effect of ruscogenin on NLRP3 mRNA levels in PA-induced HUVECs. (C) Effect of ruscogenin on TNXIP and NLRP3 protein levels in PA-induced HUVECs; ** $p < 0.01$, # $p < 0.05$, ### $p < 0.01$

DISCUSSION

Ruscogenin, a major bioactive steroid sapogenin, is found in the roots of the traditional Chinese herb, *Ophiopogon japonicus*, that has been shown to alleviate lipopolysaccharide-induced lung inflammation [10], inhibit oxidative stress and inflammatory diseases [11]. Since endothelial inflammation is considered a hallmark of cardiovascular diseases, the effect of ruscogenin on endothelial inflammation and its possible protective effect against cardiovascular diseases were investigated in this study.

Endothelial cells generally undergo apoptosis after exposure to elevated PA through stimulation of the mitogen-activated protein kinase (MAPK) pathway [12].

PA induces endothelial dysfunction, decreases cell viability, and enhances apoptosis of HUVECs, and used here as a model to investigate mechanisms involved in eicosapentaenoic acid-mediated atherogenesis [13]. The results confirm that PA decreases HUVECs viability and promotes their apoptosis. Ruscogenin has been shown to reduce lipopolysaccharide-induced endothelial cell apoptosis [14]. The present findings demonstrated that ruscogenin exerted an anti-

cytotoxic effect in PA-induced HUVECs as evidenced by decreased apoptosis and increased cell viability.

PA can induce endothelial cell apoptosis by enhancing TNF- α expression and ROS accumulation [15]. Moreover, IL-1 β and TNF- α function as pro-inflammatory cytokines, and ICAM-1 and MCP-1 function as adhesion molecules that promote endothelial inflammation [16]. Therefore, in line with a previous study that showed PA induced endothelial inflammation by increasing IL-1 β , TNF- α , ICAM-1, and MCP-1 [17], endothelial inflammation by PA was also demonstrated in this study.

Ruscogenin can reportedly exert anti-inflammatory effects by decreasing TNF- α , ICAM-1, and MCP-1 [8,9,14]. We also showed that pretreatment with ruscogenin dose-dependently inhibited PA-induced inflammation and promoted a suppressive effect on IL-1 β , TNF- α , ICAM-1 and MCP-1 secretion. ROS accumulation and reduced nitric oxide levels are major contributors to endothelial dysfunction [18]. PA-induced ROS accumulation and low nitric oxide, aggravate endothelial inflammation [17], and the role of ruscogenin on ROS and nitric oxide accumulation in PA-induced HUVECs should be investigated in future studies.

The NLRP3 inflammasome is an important innate immune system component that is activated in vascular inflammation and exacerbates inflammation by enhancing IL-1 β [19]. ROS accumulation leads to the dissociation of TXNIP from thioredoxin and promotes the binding of TXNIP to NLRP3, thus activating the NLRP3 inflammasome [20]. Therefore, inhibition of TXNIP could serve as a therapeutic strategy for inactivating inflammasomes in the setting of cardiovascular diseases [21]. Consistent with a previous report that TXNIP/NLRP3 was activated in PA-treated HUVECs [21], we observed significant increases in TXNIP and NLRP3 in PA-induced HUVECs. Ruscogenin has been shown to suppress TXNIP/NLRP3 inflammasome activation to ameliorate cerebral ischemia [22]. The results also demonstrate that TXNIP and NLRP3 were dose-dependently decreased in PA-induced HUVECs pretreated with ruscogenin. Collectively, these results indicate that ruscogenin might suppress inflammatory cytokine secretion and NLRP3 inflammasome activation to ameliorate PA-induced cytotoxicity in HUVECs. MAPK and TLR4-mediated nuclear factor (NF)- κ B signaling are also involved in PA-induced endothelial inflammation in HUVECs [23,24], and ruscogenin could also regulate these pathways [9-11,14]. Whether MAPK and NF- κ B

pathways are directly involved in ruscogenin-mediated endothelial inflammation in PA-induced HUVECs needs to be further investigated.

CONCLUSION

The findings of this study show that ruscogenin can increase cell viability but decrease the apoptosis of PA-induced HUVECs. The likely mechanism of action is the suppression of the secretion of inflammatory cytokines through inactivation of TXNIP/NLRP3 inflammasome pathways, thus ameliorating PA-triggered endothelial dysfunction. These findings suggest that ruscogenin may be an effective therapeutic agent for cardiovascular diseases.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Hongtao Liu and Xiaoli Niu designed the study, supervised the data collection, and analyzed the data; Simin Zheng interpreted the data and prepared the manuscript for publication, and Hongfei Xiong supervised the data collection, analyzed the data, and reviewed the draft of the manuscript. All authors read and approved the final manuscript.

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