Notoginsenoside Fc alleviates oxidized low-density lipoprotein-induced endothelial cell dysfunction and upregulates PPAR-γ in vitro

Authors: Yuanhao Zhang, Kui Liu and Dile Wang

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Notoginsenoside Fc alleviates oxidized low-density lipoprotein-induced endothelial cell dysfunction and upregulates PPAR-γ in vitro

Running Head: The roles of NFc in HUVECs

Yuanhao Zhang, Kui Liu, Dile Wang*
Department of Vascular Surgery, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430014, China.

*Corresponding author: The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, No.26 Shengli Street, Jiang ‘an District, Wuhan, China. E-mail: newking16888@163.com

Abstract

Background: Deep venous thrombosis (DVT) is a prevalent vascular disease and a major cause of morbidity and mortality worldwide. Notoginsenoside Fc (NFc) is a protopanaxadiol-type saponin that has been shown to have beneficial effects on several disorders. However, its function in DVT is unclear.

Methods: Human umbilical vein endothelial cells (HUVECs) were treated with oxidized low-density lipoprotein (ox-LDL) to mimic DVT in vitro and treated with NFc to investigate its functions. CCK-8 assay was utilized for measuring cell viability. Western blotting was used for detecting protein levels of proinflammatory cytokines, apoptosis-related markers, and peroxisome proliferator-activated receptor-γ (PPAR-γ). Flow cytometry was performed for cell apoptosis detection. Levels of oxidative stress-related markers were examined by the DCFH-DA method and ELISA. RT-qPCR was utilized for the measurement of PPAR-γ mRNA level.

Results: NFc increased the viability and suppressed inflammation, apoptosis, and oxidative stress in ox-LDL-treated HUVECs. NFc treatment induced upregulation of PPAR-γ in HUVECs.

Conclusion: NFc mitigates ox-LDL-induced dysfunction of HUVECs.
Key words: deep venous thrombosis; notoginsenoside Fc; inflammation; oxidative stress; endothelial; PPAR-gamma

Introduction

Deep venous thrombosis (DVT) refers to the formation of a blood clot within a deep vein and is one of the most prevalent thromboembolic disorders (Olaf and Cooney, 2017). DVT most commonly occurs in the deep veins of the lower extremities, which is clinically manifested by edema and pain and can cause the dysfunction of lower extremities (Lou et al., 2021). Thrombus in circulating blood flow can cause pulmonary embolism (PE), a thromboembolic disease with high mortality (Giordano et al., 2017; Zhang et al., 2019). In the later stage of DVT, approximately 20-50% of patients develop post-thrombotic syndrome which adversely affects the life quality of patients (Rabinovich and Kahn, 2017). Anticoagulation is the mainstay for DVT treatment which can prevent new thrombus formation and decrease the risk of PE, however, it elevates the risk of developing post thrombotic syndrome (Herrera and Comerota, 2011). Hence, there is a pressing need to find new effective approaches for DVT treatment.

Venous stasis, endothelial dysfunction, and hypercoagulability are considered the main causes of DVT formation (Sun et al., 2020). Normal vascular endothelial cells are essential for the maintenance of vascular homeostasis, prevention of thrombosis, and regulation of inflammation (Duan et al., 2021). Activation of endothelial cells can trigger the coagulation system through the induction of tissue factor (Branchford and Carpenter, 2018). Human umbilical vein endothelial cells (HUVECs) represent a useful vascular model to study DVT at the cellular level. Oxidized low-density lipoprotein (ox-LDL) and its components exert a pivotal effect on the progression of thrombotic events (Obermayer et al., 2018). Studies have demonstrated that ox-LDL is cytotoxic and can enhance the activity of endothelial cell tissue factor to promote coagulation (Weis et al., 1991). HUVECs stimulated with ox-LDL have been used to establish the in vitro model of DVT (Pan et al., 2021). Increasing evidence has illustrated that inflammation and oxidative stress are critical factors in the pathogenesis of DVT (Ekim
et al., 2014; Borgel et al., 2019; Chu et al., 2022). Oxidative stress is caused by overproduction of reactive oxygen species (ROS) or insufficiency of the antioxidant defense system, such as downregulation of superoxide dismutase (SOD) (Wang and Zennadi, 2020).

*Panax notoginseng*, a traditional Chinese herbal medicine, has been widely used in the treatment of vascular disorders in China for its pharmacological effects on facilitating blood circulation, eliminating blood stasis, and reducing inflammation (Wang et al., 2016). Notoginsenoside Fc (NFc) is a protopanaxadiol-type saponin isolated from the leaves of *Panax notoginseng* (He et al., 2015). Emerging evidence has indicated that NFc can suppress platelet aggregation (He et al., 2015; Liu et al., 2018b). NFc can facilitate reendothelialization in a high glucose-induced diabetic rat model by enhancing endothelial cell autophagy (Liu et al., 2019). Importantly, NFc demonstrated anti-inflammatory and anti-apoptotic functions in high glucose-induced rat aortic endothelial cells (Liu et al., 2018a). Nevertheless, whether NFc can affect the progression of DVT is unclarified.

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a transcription factor belonging to the nuclear receptor superfamily (Kumar et al., 2021). PPAR-γ is implicated in the progression of many diseases, such as obesity, non-alcoholic fatty liver disease, and cancers (Vallée and Lecarpentier, 2018; Skat-Rørdam et al., 2019, Faghfouri et al., 2021). Furthermore, PPAR-γ was shown to have a protective role in aortic thrombosis by downregulating P-selection (Jin et al., 2015). *Panax notoginseng* saponins can activate the PPAR-γ pathway in a rat hypercoagulability model induced by thrombin (Shen et al., 2017). Nevertheless, it is unclear whether NFc can regulate PPAR-γ in ox-LDL-stimulated HUVECs.

Herein, we probed the functions of NFc and its potential mechanism in an *in vitro* DVT model induced by ox-LDL in HUVECs. It was hypothesized that NFc might affect cell viability, apoptosis, inflammation, and oxidative stress. The results might provide a new perspective for treating DVT.
Materials and methods

Preparation of NFc

NFc (purity ≥ 98%) was obtained from Shanghai Shifeng Biotechnology Co., Ltd (Shanghai, China) and diluted by dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) to obtain different doses of NFc (0-500 µM). The chemical structure of NFc is shown in Figure 1A.

Cell culture and treatment

HUVECs were purchased from BeNa Culture Collection (BNCC337616, Beijing, China) and incubated in endothelial cell medium (ScienCell, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Hyclone, Salt Lake City, UT, USA) and 1% penicillin/streptomycin (Sigma-Aldrich) at 37°C in a humidified incubator with 5% CO₂. The culture medium was changed every two days and cells at passage 3-5 were used for following assays.

Cells were treated with gradient doses of ox-LDL (0-80 µg/ml; YEASEN, Shanghai, China) or NFc (0-500 µM) for 48h or treated with 40 µg/ml ox-LDL for different durations (0-48h). In some experiments, cells were stimulated with 40 µg/ml ox-LDL to mimic DVT in vitro (Pan et al., 2021) and/or treated with 20 µM NFc to analyze its effects on endothelial cell injury (Liu et al., 2018a).

Cell counting kit-8 (CCK-8) assay

HUVECs were inoculated into 96-well plates (1×10⁴ cells/well) and incubated under indicated treatments for 48h, followed by adding 10 µl of CCK-8 solution (Beyotime, Shanghai, China) to each well and incubation for another 2h. Afterwards, cell viability was analyzed by measuring the optical density at 450 nm using a microplate reader (Olympus, Tokyo, Japan).

Western blotting

Proteins were isolated from indicated HUVECs using RIPA buffer (Thermo Scientific, Waltham, MA, USA) and quantified using a BCA assay kit (Bio-Rad, Hercules, CA,
USA). Protein samples (20 µg) were resolved in 10% SDS-PAGE and blotted on polyvinylidene fluoride membranes (Bio-Rad). Afterwards, the membranes were blocked with 5% defatted milk, incubated at 4°C overnight with primary antibodies against: tumor necrosis factor alpha (TNF-α; ab183218, 1:1000), interleukin (IL)-1β (ab254360, 1:1000), IL-6 (ab233706, 1:1000), intercellular adhesion molecule-1 (ICAM-1; ab282575, 1:1000), Bax (ab32503, 1:1000), Bcl-2 (ab32124, 1:1000), Cleaved (C)-caspase3 (ab32042, 1:500), PPAR-γ (ab272718, 1:1000), GAPDH (ab181602, 1:10000) (all from Abcam, Cambridge, MA, USA), and then incubated with the goat anti-rabbit secondary antibody (ab97080, Abcam) at room temperature for 2h. Eventually, protein bands were visualized with an enhanced chemiluminescence reagent (Thermo Scientific) and quantified with ImageJ software (NIH, Bethesda, CA).

Flow cytometry
Cell apoptosis was measured by flow cytometry using Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). HUVECs under indicated treatments were washed with PBS, centrifuged, and resuspended in 100 µl 1× binding buffer. Next, cells were incubated with 5 µl Annexin V-FITC and 10 µl propidium iodide solution for 10 mins at room temperature away from light. A total of 400 µl 1× binding buffer was added. Cell apoptosis was analyzed using a flow cytometer (BD Biosciences).

Measurement of oxidative stress-related markers
The production of ROS in HUVECs was measured by a 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe (2 µL, 10 mM; Beyotime). Cells with indicated treatments were incubated in a culture medium containing 0.1% fluorescent DCFH-DA at 37°C for 30 mins in the dark. (Yang et al., 2020). Then, cells were resuspended in PBS and evaluated by FACSCalibur flow cytometer (BD Biosciences). The percentage of ROS-positive cells was measured with FlowJo software 10.4 (TreeStar, Ashland, OR, USA). The concentrations of malondialdehyde
(MDA) and SOD were assessed by enzyme-linked immunosorbent assay (ELISA) using a human MDA ELISA kit (ab287797, Abcam) and human SOD ELISA kit (ab119520, Abcam), respectively, following the manufacturer’s protocols.

**Real-time quantitative polymerase chain reaction (RT-qPCR)**

Total RNA was extracted from HUVECs using TRIzol reagent (Invitrogen, Carlsbad, C, USA). Approximately 1 µg of total RNA was reverse transcribed using iScript cDNA Kit (Bio-Rad) to obtain cDNA. RT-qPCR was conducted on an ABI PRISM 7300 system using SYBR Premix Ex Taq™ (Takara, Dalian, China). Relative expression of PPAR-γ was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to GAPDH. Primer sequences are listed as follows:

**PPAR-γ**
- Forward: 5’-TTCCATTTCACAGACAGATCC-3’
- Reverse: 5’-CTTTGATTGCACTTTGGTACTC-3’

**GAPDH**
- Forward: 5’-TCAAGATCATCAGCAATGCC-3’
- Reverse: 5’-CGATACCAAAGTTGTCATGGA-3’

**Statistical analysis**

Data are presented as the mean ± standard deviation (SD) and were analyzed using SPSS 21.0 software (IBM, Armonk, NY, USA). Difference comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc analysis. The value of $p<0.05$ was considered statistically significant.

**Results**

**NFC attenuates ox-LDL-induced cytotoxicity in HUVECs**

To determine the effect of ox-LDL on cell viability, HUVECs were stimulated with different concentrations of ox-LDL (0-80 µg/ml) for 48h. Notably, ox-LDL dose-dependently decreased HUVEC viability, with a significant decrease in cell viability at 40 and 80 µg/ml, as displayed by CCK-8 assay (Fig. 1B). Subsequently, HUVECs were
treated with ox-LDL (40 µg/ml) for different durations (0-48h) and the results from Fig. 1C presented that ox-LDL time-dependently reduced HUVEC viability. These results confirmed the cytotoxicity of ox-LDL to HUVECs. Moreover, it was found that NFc significantly impaired HUVEC cell viability at 500 µM and was not cytotoxic at concentrations of 1, 10, 20, 50, and 100 µM (Fig. 1D). Then, NFc at concentrations of 0-100 µM was used to treat HUVECs in the presence of 40 µg/ml ox-LDL. As shown in Fig. 1E, NFc attenuated ox-LDL-mediated inhibition of cell viability, with 20 µM NFc showing the most significant effect. Thus, 20 µM NFc was selected as an optimal dose for subsequent experiments. After that, HUVECs were treated with NFc (20 µM) and ox-LDL (40 µg/ml) for different durations (0-48h), and the results displayed that NFc time-dependently abated the suppressive effect of ox-LDL on cell viability (Fig. 1F). Collectively, these data revealed that NFc could attenuate ox-LDL-induced cytotoxicity in HUVECs.

NFc reduces the expression of inflammatory cytokines in ox-LDL-treated HUVECs

Inflammatory response is a pivotal factor in the pathophysiological process of DVT (Chu et al., 2022). Hence, we tested NFc effect on inflammation in HUVECs. HUVECs were stimulated with NFc (20 µM) and/or ox-LDL (40 µg/ml) for 48h and levels of proinflammatory factors were examined. Notably, ox-LDL markedly elevated protein levels of the proinflammatory factors (TNF-α, IL-6, IL-1β and ICAM-1) in HUVECs, whereas this effect was alleviated in NFc and ox-LDL co-treatment group (Fig. 2A-E), indicating that NFc has an anti-inflammatory role in ox-LDL-treated HUVECs.

NFc suppresses ox-LDL-mediated HUVEC apoptosis

The impact of NFc on HUVEC apoptosis was also investigated. Results from flow cytometry displayed that NFc markedly suppressed ox-LDL-mediated enhancement of the percentage of apoptotic cells (Fig. 3A-B). Consistently, western blotting showed that ox-LDL enhanced levels of pro-apoptotic proteins (Bax, C-caspase3) while it reduced levels of the anti-apoptotic protein (Bcl-2) in HUVECs (Fig. 3C-F), indicating
that ox-LDL induces HUVEC apoptosis. Notably, NFc treatment significantly abated the pro-apoptotic effect of ox-LDL on HUVECs (Fig. 3C-F).

**NFc reduces ox-LDL-mediated ROS production in HUVECs**

Oxidative stress caused by ROS overproduction is another critical factor in the progression of DVT (Yang et al., 2019). We tested whether NFc impacted oxidative stress in ox-LDL-treated HUVECs. DCFH-DA method was utilized for measuring the production of ROS. As shown by the results, NFc significantly reduced ROS production (Fig. 4A-B), indicating the antioxidant role of NFc. To further substantiate this, we examined the levels of MDA and SOD in HUVECs. As expected, NFc offset ox-LDL-triggered increase in MDA level and reduction in SOD level (Fig. 4C-D). These results indicated that NFc could reduce ox-LDL-induced ROS production in HUVECs.

**NFc induces upregulation of PPAR-γ in HUVECs**

To probe the potential mechanism of NFc in affecting HUVEC functions, we analyzed its relationship with PPAR-γ. The downregulation of PPAR-γ was observed in HUVECs under treatment of increasing doses of ox-LDL (Fig. 5A-C), indicating that PPAR-γ might play a potential role in DVT. Then, the expression of PPAR-γ was examined in HUVECs treated with NFc and/or ox-LDL. As shown in Fig. 5D-F, NFc enhanced PPAR-γ expression at both mRNA and protein levels in HUVECs stimulated by ox-LDL. These data revealed that NFc could upregulate PPAR-γ in ox-LDL-treated HUVECs.

**Discussion**

DVT is a life-threatening complication after surgery, related to high morbidity and mortality (Khoury et al., 2020). Despite advances in therapy techniques, the incidence of DVT is still high and many patients are afflicted with post thrombotic syndrome (Wang et al., 2021). Hence, better understanding the underlying mechanism and finding novel effective approaches for DVT treatment are of great significance. Ox-LDL-mediated endothelial dysfunction is implicated in the pathogenesis of a variety of
vascular disorders, including DVT (Pan et al., 2021). Based on the evidence, an in vitro DVT model was established by ox-LDL induction in HUVECs to probe the functions of NFc in DVT.

NFc is a protopanaxdiol-type saponin that has been shown to inhibit platelet aggregation by repressing phospholipase Cγ2 and subsequent DAG-PKC-TXA2 and IP3-[Ca2+], and platelet aggregation plays a critical role in the formation of blood clots (Liu et al., 2018b). NFc was shown to play a protective role in vascular injury in high-glucose-induced diabetic rats (Liu et al., 2018a, Liu et al., 2019). These indicated the potential role of NFc in vascular diseases. However, the detailed functions of NFc in ox-LDL-induced DVT are unclear. Here, we found that NFc dose-dependently and time-dependently increased the viability of HUVECs. NFc exhibited no cytotoxicity at the concentration of 100 µM, but it significantly impaired HUVEC cell viability at 500 µM. Moreover, growing evidence has indicated that inflammation plays a pivotal role in the pathogenesis of DVT and is closely associated with coagulation (Borgel et al., 2019). To identify NFc impact on ox-LDL-induced inflammation in HUVECs, we examined the levels of proinflammatory cytokines. The results from the study displayed that NFc markedly reduced the production of TNF-α, IL-6, IL-1β and ICAM-1 in ox-LDL-treated HUVECs. ICAM-1, a transmembrane cell surface glycoprotein, results in the adhesion of leukocytes to the vascular wall, which consequently leads to inflammation (Soveyd et al., 2018). These indicated the anti-inflammatory role of NFc in HUVECs. Furthermore, the impact of NFc on cell apoptosis and oxidative stress was also probed. The results displayed that NFc significantly alleviated ox-LDL-evoked apoptosis of HUVECs, as evidenced by increased Bcl-2 protein level and decreased Bax and C-caspase3 protein levels, confirming the anti-apoptotic effect of NFc. The imbalance between antioxidant and oxidant systems is termed as oxidative stress, resulting from overproduction of ROS and/or insufficiency of antioxidant defense mechanism (Ekim et al., 2014). MDA, a product of lipid peroxidation, can impair the antioxidant defense system and induce cell apoptosis (Bergin et al., 2021). SOD is an antioxidant enzyme which can counteract oxidative stress and protect against lipid peroxidation, protein oxidation and DNA damage (McCord and Edeas, 2005).
Increasing evidence has demonstrated that oxidative stress impairs red blood cell quality and function, elicits endothelial dysfunction, and activates platelets and leukocytes, consequently affecting the clotting system and leading to DVT (Ekim et al., 2014, Wang and Zennadi, 2020). Here, we found that NFc markedly decreased the production of ROS and MDA while it increased the level of SOD in ox-LDL-treated HUVECs, indicating that NFc attenuated ox-LDL-induced oxidative stress. Collectively, the above results indicate that NFc can attenuate the dysfunction of HUVECs induced by ox-LDL.

Previous studies have elucidated that PPAR-γ is implicated in multiple human diseases, including aortic thrombosis (Jin et al., 2015). PPAR-γ was shown to have an anti-inflammatory role, and downregulation of PPAR-γ was observed during oxidative stress and inflammation (Vallée and Lecarpentier, 2018, Vetuschi et al., 2018). Additionally, the PPAR-γ pathway was reported to be activated by Panax notoginseng saponins in a rat hypercoagulability model induced by thrombin (Shen et al., 2017). In the present study, we examined whether NFc could affect PPAR-γ expression in DVT in vitro. The results presented that NFc could offset ox-LDL-triggered downregulation of PPAR-γ, indicating that the protective effect of NFc on DVT may be associated with PPAR-γ upregulation. However, future studies are needed to elucidate our findings.

In conclusion, we probed the functions of NFc in DVT in vitro. The results reveal that NFc increases the viability and suppresses inflammatory response, oxidative stress and apoptosis of HUVECs. Additionally, NFc upregulates PPAR-γ expression in HUVECs. These findings may provide a new credible perspective for treating DVT. In vivo studies are needed to further investigate the protective role of NFc in DVT. Moreover, considering the complexity of mechanisms, further research may benefit from exploring potential signaling pathways mediated by NFc in DVT, such as anti-inflammatory and antioxidant signaling pathways.

**Declarations**

**Conflicting interests**

The authors have no conflicts of interest to declare.
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None

Ethical approval
Our study did not require an ethical board approval because it did not contain human or animal trials.

Guarantor
DW

Contributorship
KL and YZ conceived and designed the experiments. DW, KL and YZ carried out the experiments. DW, KL and YZ analyzed the data. DW, KL and YZ drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

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**Figure legends**

**Figure 1.** NFc protects from ox-LDL-induced cytotoxicity in HUVECs. A. Chemical structure of NFc. B. CCK-8 assay for assessing the viability of HUVECs treated with different doses of ox-LDL (0-80 µg/ml) for 48h. C. Viability of HUVECs treated with 40 µg/ml ox-LDL for different durations (0-48h). D. Viability of HUVECs exposed to different doses of NFc (0-500 µM) for 48h. E. HUVEC viability under treatment of NFc (0-100 µM) in the presence of 40 µg/ml ox-LDL for 48h. F. HUVEC viability under treatment of 20 µM NFc and 40 µg/ml ox-LDL for different durations (0-48h). *p<0.05, **p<0.01, ***p<0.001.

**Figure 2.** NFc reduces the expression of inflammatory cytokines in ox-LDL-treated HUVECs. A. Western blotting of proinflammatory factors in HUVECs with or without treatment of 20 µM NFc and/or 40 µg/ml ox-LDL for 48h. B-E. Quantification of protein levels of these proinflammatory factors. **p<0.01, ***p<0.001.

**Figure 3.** NFc suppresses ox-LDL-mediated HUVEC apoptosis. A-B. Flow cytometry for assessing HUVEC apoptosis. C. Western blotting of apoptosis-associated proteins in indicated HUVECs. D-F. Quantification of levels of Bax, Bcl-2, and C-caspase3. **p<0.01, ***p<0.001.

**Figure 4.** NFc reduces ox-LDL-mediated ROS production. A. DCFH-DA method for evaluating ROS production in HUVECs. B. Quantification of ROS-positive cells by flow cytometry. C-D. Concentrations of MDA and SOD in HUVECs determined by ELISA. **p<0.01.
Figure 5. NFc upregulates PPAR-γ in HUVECs. A-C. RT-qPCR and western blotting for assessing PPAR-γ mRNA and protein expression in HUVECs treated with increasing doses of ox-LDL. D-F. PPAR-γ mRNA and protein levels in HUVECs with or without treatment of 20 µM NFc and/or 40 µg/ml ox-LDL for 48h. **p<0.01, ***p<0.001.