doi: 10.4149/gpb_2019062

Angiopoietin-like protein 7 mediates TNF-α-induced adhesion and oxidative stress in human umbilical vein epithelial cells

Jiong Li[1,2,*], Tianxiang Liang[2,*], Yanzheng Wang[2], Yirong Gan[2], Zongke Kou[2], Yunlong Zhang[2], Rui Mao[2] and Dingxiong Xie[2,3]

[1] *Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Lanzhou University, Lanzhou 730000, China*
[2] *Gansu Cardiovascular Institute, Lanzhou 730050, China*
[3] *Lanzhou University Second Hospital, Lanzhou 730030, China*

Abstract. Tumor necrosis factor-α (TNF-α) promotes monocyte adhesion to endothelium and accumulation of endothelium will lead to atherosclerosis. The present study explored angiopoietin-like protein (Angptl7) as a potential target in the process of atherosclerosis, and its role in the adhesion and oxidative stress induced by TNF-α in human umbilical vein epithelial cells (HUVEC). The initiation of atherosclerosis is endothelial injury. Angptl7 was dramatically increased in TNF-α-induced HUVEC compared to the control cells. After Angptl7 effectively knocked-down in TNF-α-induced HUVEC, the levels of reactive oxygen species (ROS), interleukin (IL)-1β, IL-6 and cyclooxygenase-2 (Cox-2) were prominently decreased, whereas the levels of nitric oxide (NO) and endothelia nitric oxide synthase (eNOS) were increased. Inhibition of Angptl7 significantly reversed TNF-α-induced cell adhesion in HUVEC. Finally, downregulation of Angptl7 significantly reduced the expression of nuclear factor-κB (NF-κB) and enhanced the levels of nuclear factor erythroid 2-related factor 2 (Nrf-2) and heme oxygenase-1 (HO-1) in TNF-α-treated HUVEC. Angptl7 conducted TNF-α-induced oxidative stress and cell adhesion in HUVEC. Therefore, Angptl7 might participate in the development of endothelial injury and further atherosclerosis. This might give us a new insight for investigation of procession of atherosclerosis.

Key words: Angiopoietin-like protein 7 — Atherosclerosis — Cell adhesion — Oxidative stress — Inflammation

Introduction

Atherosclerosis is the most common cause of cardiovascular disease. Preclinical assessment of changes in vascular endothelial cells plays a pivotal role in improving cardiovascular risk (Onat et al. 2011). Endothelial cell dysfunction exists in the injurious lesion of arterial blood vessels, which is an early marker of atherosclerotic injury (Virmani et al. 2000; Bar et al. 2019). Vascular endothelium is a layer of monocytes on the inner walls of blood vessels, regulating vascular wall function *via* autocrine and paracrine system. Human vascular endothelial dysfunction is caused by oxidative stress and promoted inflammatory signaling in endothelial cells (Migrino et al. 2017). Importantly, vascular inflammation is a main initial effect to induce atherosclerosis that is a complex vascular injury. The vascular plaques are formed by the interaction of plasma lipids with vascular wall and immune cells (Forstermann et al. 2017). In addition, tumor necrosis factor-α (TNF-α) promotes monocyte adhesion to the endothelium (Ende et al. 2014), and stimulates expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Li et al. 2019). The aggregation of endothelial cells to monocytes contributes to the inflammatory response and the development of atherogenesis (Napoleone et al. 1997).

* These authors have the similar contribution to this investigation. Correspondence to: Dingxiong Xie, Gansu Cardiovascular Institute, No. 1 of Wujiayuan West Street, Qilihe District, Lanzhou 720050, China
E-mail: xiedingxiong45@126.com

Elevation of reactive oxygen species (ROS), inflammation, high levels of free fatty acids (FFAs) and reduction of NO are classical hallmarks of endothelial dysfunction.

A variety of studies indicated that transcription factor, nuclear factor-κB (NF-κB), participates in the induction of adhesion molecules (VCAM-1, ICAM-1) in inflammatory experiments (Bhaskar et al. 2016). TNF-α induces ROS *via* endothelial mitochondria and activated NF-κB and secretion of IL-6 (Schulze-Osthoff et al. 1993). NO is formed by metabolization of L-arginine *via* endothelial isoform of nitric oxide synthase (eNOS) (Ye et al. 2019). Generation of ROS and reduction of NO in endothelial cell are key events in atherosclerosis (Forstermann et al. 2017). Activation of nuclear factor erythroid 2-related factor 2 (Nrf-2) suppresses oxidative stress in vascular endothelial cells (Zhu et al. 2019) by binding to antioxidant response element (ARE), including quinone oxido-reductase 1 (NQO1) (Yang et al. 2019), heme oxygenase-1 (HO-1) (Kapoor et al. 2019), and the expression of toll-like receptor 4 (TLR4) (Shan et al. 2019).

Angiopoietin-like (Angptls) proteins have been identified to include a coiled-coil domain and a fibrinogen-like domain which is similar to angiopoietins. Up to now, 7 proteins have been identified as members of the Angptls family. As a secreted glycoprotein, Angiopoietin-like protein 7 (Angptl7) could reduce tumor growth and aberrant blood vessel formation (Parri et al. 2014). Recent research indicated that Angptl7 contributes to AngII-induced proliferation, inflammation and apoptosis in vascular smooth muscle cells (Zhao et al. 2019). In current study, we employed cultured human endothelial cells to explore the roles of Angptl7 in TNF-α-induced adhesion and oxidative stress, and to elucidate the further mechanism.

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from China Center for Type Culture Collection (Wuhan, China) and cultured in minimum essential medium (MEM Eagles with Earle's Balanced Salts; Gibco, ThermoFisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, ThermoFisher Scientific, USA). The cells were incubated in a 37°C, 5% CO_2 atmosphere. The cells were transfected with siRNA-Angptl7 (si-Angptl7) and siRNA control (si-NC) in HUVEC. The sequences of siRNA-1 targeting ANGPTL7 were sense: 1 5′-GCACAAGACACCAGCACA Gtt-3′ and anti-sense 5′-CUGUGCUGGUGUCUUGUG Ctt-3′. The siRNA-Angptl7 and negative control were purchased from GenePharma (Shanghai, China). Transfection of si-Angptl7 and si-NC was performed using Lipofectamine 2000 (Invitrogen) according to manual's protocol.

Cell counting kit-8 (CCK-8)

To induce an adhesion and oxidative stress in HUVEC, cell viability of HUVEC was determined by CCK-8 assay kit (Glpbio Technology, China) according to the manufacturer's introductions. In brief, the cells were seeded at a concentration of 1×10^4 cells suspended in 100 μl culture medium each well. After cells were attached to plate, they were treated with TNF-α (0, 1, 5, 10, 20, 40, 80 ng/ml) and incubated for 48 h. Subsequently, the absorbance data were obtained by Varioskan LUX Multimode Microplate Reader (Thermofisher Scientific, USA).

Western blotting

The Western blotting was performed to detect the protein levels according to previous investigation (Pan 2017). Angptl7 transfected and un-transfected HUVEC were treated with TNF-α (40 ng/ml) for 48 h with a density of 8×10^5 cells/well in 6-well plates. The following antibodies were used: Angptl7, eNOS, NF-κB, Nrf-2, HO-1, GAPDH, and HRP-conjugated secondary antibodies. The immune-blotting was visualized using BioSpectrum Gel Imaging System (Bio-Rad, USA) and analyzed by Image J 1.8.0.

RT-PCR

Total RNA was extracted and purified using RiboPure™ RNA Purification Kit (Invitrogen; ThermoFisher Scientific). Subsequently, cDNA was synthesized from purified total RNA using SuperScript™ IV First-Strand Synthesis System (Invitrogen; ThermoFisher Scientific). Finally, SYBR™ Green PCR Master Mix (Applied Biosystems; ThermoFisher Scientific) was performed to detect gene expression. All assay kits were used in line with the manufacturer's instructions. QuantStudio 2 Real-Time PCR System (Applied Biosystems; ThermoFisher Scientific) was used to detect and analyze the expression levels of gene.

Reactive oxygen species (ROS) production

ROS was measured by ROS assay kit (Beyotime Biologic Technology, China) according to the manual's protocol. In brief, cells (4×10^5 cells/well) seeded in 6-well plates were treated as described in section *Western blotting*. The cells were resuspended in a serum-free culture medium containing 2',7'-dichlorofluorescein diacetate (DCFH-DA) detection probe. The fluorescent intensity in different groups was detected using a flow cytometer (Becton-Dickinson, USA).

NO production

Cells (4×10^5 cells/well) were treated as described in section *Western blotting*. The culture medium was collected and NO in different groups was quantified by the Griess method according to the manual's protocol (Beyotime Biologic Technology, China).

The detection of inflammatory factors by ELISA

Cells (4×10^5 cells/well) were treated as described in section *Western blotting*. Levels of IL-1β (ab100562, Abcam), IL-6 (ab178013, Abcam), Cox-2(DYC4198-2, R&D systems) in culture medium were measured using commercial assay kit according to manual. The data were detected by Varioskan LUX Multimode Microplate Reader (Thermofisher Scientific, USA).

Cell adhesion assay

Cells were digested to cells suspension and diluted into proper concentration with medium. Cells suspension in TNF-α-treated group was added with TNF-α in 10 ng/ml. 5×10^6 cells were added in each well of a 12-well plates. Cells were transfected with si-NC or si-Angptl7 and both treated with TNF-α (TNF-α+si-NC and TNF-α+si-Angptl7 group) to evaluate the effects of Angptl7 in TNF-α-induced cells. These two groups were processed in the similar way like TNF-α-treated group, but the cells were transfected with shRNA in advance. After 8 h, cells were washed with PBS and stained with DAPI (ThermoFisher Scientific, USA). Image J 1.8.0 was performed to measure the adhesion cells.

Statistical analysis

Data were presented as mean ± standard deviation (SD). Multiple comparisons were performed using one-way ANOVA analysis following with Turkey's test. Difference between groups was determined by Student's *t*-test. A *p*-value < 0.05 was considered as a statistical difference.

Results

TNF-α induced expression of Angptl7 in HUVEC

TNF-α is usually highly expressed in atherosclerotic dysfunction and causes a variety of vascular diseases. More than 10 ng/ml of TNF-α resulted in toxicity for HUVEC, and 40 ng/ml of TNF-α led to approximately 50% cell death (Fig. 1). Since some effects of Angptl7 in atherosclerosis have been found in previous investigation, we detected the expression of Angptl7 in TNF-α-induced HUVEC. The protein and mRNA levels of Angptl7 were prominently reinforced in TNF-α-induced HUVEC compared with the normal control (Fig. 2A and B). Then cells were transfected with Angptl7 shRNA and control shRNA, and all groups were induced by TNF-α. Angptl7 levels were drastically decreased in si-Angptl7-1 group (Fig. 2C and D), thus it was chosen for the following experiment.

TNF-α-induced oxidative stress and inflammation were mediated by Angptl7

TNF-α has been reported to induce HUVEC oxidative stress. To investigate the role of Angptl7 in TNF-α-induced oxidative stress, we evaluated the levels of ROS, NO and eNOS. Results exhibited that the generation of ROS induced by TNF-α was significantly reduced by inhibition of Angptl7 in HUVEC (Fig. 3A). TNF-α-impaired production of NO was significantly reversed by inhibiting Angptl7 in HUVEC (Fig. 3B). Inhibition of Angptl7 abrogated the TNF-α-induced downregulation of eNOS, which catalyzed the production of NO (Fig. 3C). In addition, silencing Angptl7 diminished TNF-α-induced expression of Cox-2, IL-1β, and IL-6 (Fig. 3D–F).

TNF-α-induced cell adhesion was mediated by Angptl7

Exogenous TNF-α significantly enhanced cell adhesion of HUVEC (Fig. 4) which is consistent with previous studies. However, cutting down the expression of Angptl7 dramatically reduced the adhesion cells (Fig. 4), indicating that Angptl7 might play a role in TNF-α-induced cell adhesion.

Effect of Angptl7 on NF-κB, Nrf-2 signaling in HUVEC

The expression levels of NF-κB were prominently increased induced by TNF-α, whereas Nrf-2 and HO-1

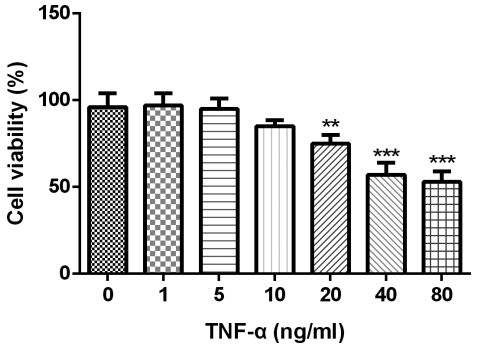

Figure 1. Cell viability of HUVEC treated with TNF-α. Cells were treated with various concentrations of TNF-α (0, 1, 5, 10, 20, 40, 80 ng/ml) for 24 h. Cell viability was detected by CCK-8 analysis. Data were represented by mean ± SD. ** $p < 0.01$, *** $p < 0.001$ *vs.* 0 ng/ml TNF-α.

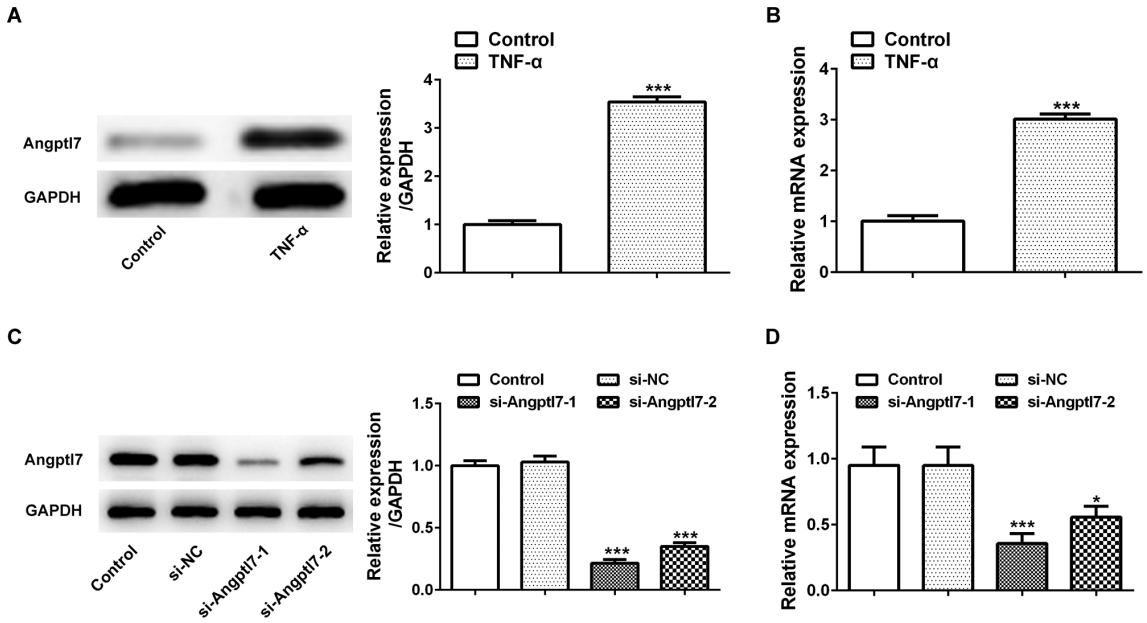

Figure 2. TNF-α induced expression levels of Angptl7 in HUVEC. The expression of Angptl7 (**A** and **B**) and the transfection efficacy of Angptl7 (**C** and **D**), both induced by TNF-α (40 ng/ml), were detected by Western blotting (on the left) and mRNA (on the right). The two silenced sequences (si-Angptl7-1 and si-Angptl7-2) were respectively used to knockdown the Angptl7 expression and sequence 1 named si-Angptl7-1 was used in the following experiment. Data were represented by mean ± SD. * $p < 0.05$, *** $p < 0.001$ *vs*. Control.

were decreased in TNF-α-induced HUVEC compared to the control (Fig. 5). Downregulation of Angptl-7 reversed the effects on TNF-α-induced alteration of NF-κB, Nrf-2 and HO-1 (Fig. 5). Therefore, TNF-α-induced activation of NF-κB and inhibition of Nrf-2/HO-1 may be mediated by Angptl7.

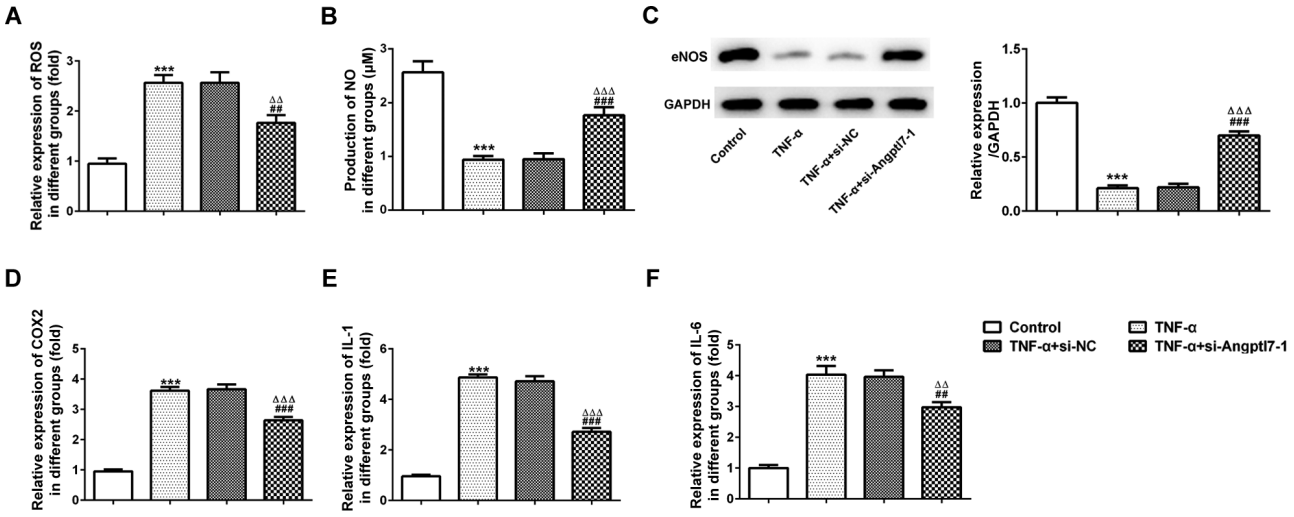

Figure 3. TNF-α induced oxidative stress and inflammation mediated by Angptl7 in HUVEC. ROS production (**A**), NO (**B**), Cox-2 (**D**), IL-1β (**E**) and IL-6 (**F**) levels were detected by ELISA analysis in different groups. **C.** The expression levels of eNOS was determined by Western blotting. TNF-α, 40 ng/ml for 24 h. Data were represented by mean ± SD. *** $p < 0.001$ compared to control; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ *vs*. TNF-α; $^{\Delta\Delta\Delta}$ $p < 0.001$ *vs*. TNF-α+si-NC.

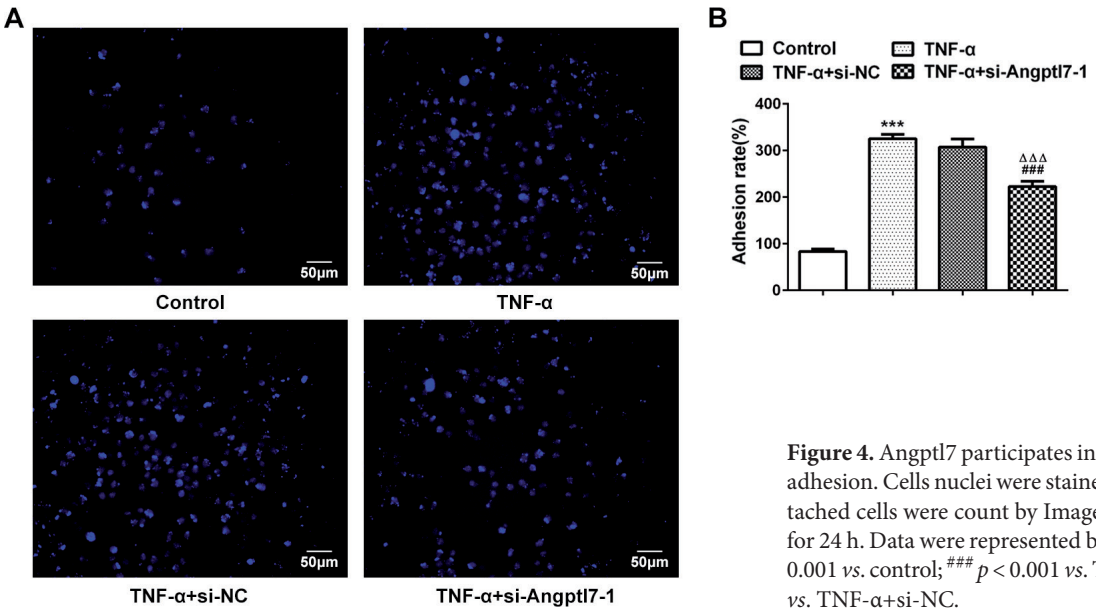

Figure 4. Angptl7 participates in TNF-α-induced cell adhesion. Cells nuclei were stained with DAPI and attached cells were count by Image J. TNF-α, 40 ng/ml for 24 h. Data were represented by mean ± SD. *** $p < 0.001$ *vs*. control; ### $p < 0.001$ *vs*. TNF-α; ΔΔΔ $p < 0.001$ *vs*. TNF-α+si-NC.

Discussion

Absence of normal endothelial function is a critical risk in atherosclerosis. Inflammation is vital in the progression of atherosclerosis, and it usually leads to endothelial dysfunction (Dinh et al. 2014). Moreover, the inflammatory factor TNF-α has been found to induce extracellular matrix deposition on arterial walls, resulting in the increase of IL-1β and IL-6 (Tsutamoto et al. 2000; Zhou et al. 2018). Inflammation and dysregulation in endothelial and vascular smooth muscle cells play critical roles in plaque formation and rupture (Rai and Agrawal 2017). The salient discovery in the present study is that Angptl7 plays a role in the TNF-α-induced endothelial dysfunction including oxidative stress, inflammation and adhesion.

A growing evidence indicates that selectively targeting oxidative stress can be an effective therapeutic modality for

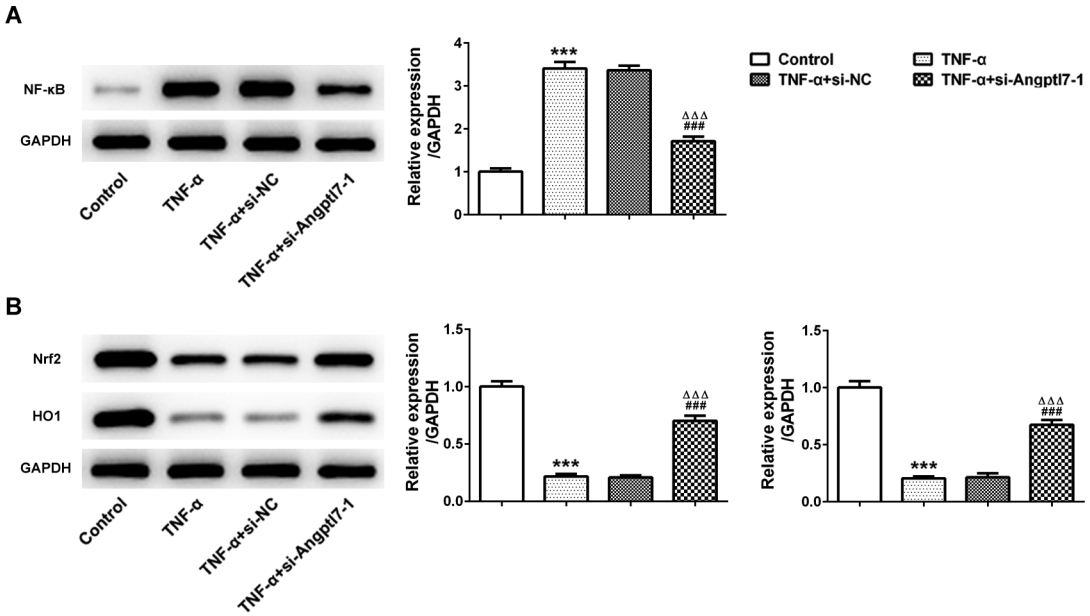

Figure 5. Angptl7 regulates TNF-α-induced NF-κB activation and Nrf-2/HO-1 inhibition. The expression of NF-κB (**A**), Nrf-2 and HO-1 (**B**) was detected by Western blotting. TNF-α, 40 ng/ml for 24 h. Data were represented by mean ± SD. *** $p < 0.001$ *vs*. control; ### $p < 0.001$ *vs*. TNF-α; ΔΔΔ $p < 0.001$ *vs*. TNF-α+si-NC.

atherosclerosis (Kattoor et al. 2017). In the present study, Angptl7 significantly alleviated TNF-α-induced ROS levels and promoted NO production, indicating that Angptl7 performed effective role in oxidative stress. Continuous release of NO in endothelium of blood vessels acts a solid part in cardiovascular disease, due to inhibiting vascular smooth muscle cell proliferation, reducing platelet activation and assembly, and abrogating atherosclerosis (Yang et al. 2015). Endothelial NO generation is catalyzed by eNOS and enzymatic cofactor tetrahydrobiopterin (Yu and Liu 2018). Therefore, Angptl7 might alleviate the catalytic effects of eNOS to reduce NO generation.

Angptl7 promotes inflammation in macrophages through p38 MAPK signaling pathway, and injections of TNF-α promotes the expression of Angptl7 in mouse eyes (Qian et al. 2016). In the current study, Angptl7 was upregulated in TNF-α-induced HUVEC. Furthermore, inhibition of Angptl7 reduced the TNF-α-induced generation of IL-1β, IL-6 and Cox-2. Downregulation of NF-κB inhibits inflammation to exert atheroprotective effects (Shen et al. 2019). Reduction of inflammation and adhesion in endothelial might contribute to reduction of atherosclerosis (Tsujikawa et al. 2019). The current study revealed that inhibition of Angptl7 could decrease the expression of NF-κB. Endothelial regulates leukocyte recruitment to exert pro-inflammatory effects, including promoting the expression of ICAM-1, IL-6, IL-8, and Céx-2 (Kuldo et al. 2005). HUVEC adhesion to monocytes has been indicated as an initiator of inflammatory vascular diseases including atherosclerosis (Xu et al. 2019). Our study preliminarily showed that inhibition of Angptl7 could reduce cell adhesion of HUVEC. However, more evidence is still needed for further study on the adhesion of HUVEC in atherosclerosis.

The Nrf-2/HO-1 signaling pathway is one of the key mechanisms in cellular anti-oxidative effects. Nrf-2 directly regulates ROS mediated by antioxidant defense systems through various mechanisms including induction of stress response proteins (HO-1) (Ma 2013). The present study showed that inhibition of Angptl7 reactivated the Nrf/HO-1 signaling pathway, indicating that it might mediate Angptl7 triggered effects in HUVEC. In addition, activation of Nrf-2 plays a role in the protection of atherosclerosis (Kim et al. 2012). However, further investigations still require deep exploration.

Conclusions

Our results firstly showed the role of Angptl7 in TNF-α-induced inflammatory factors release, disorder production of NO and ROS and adhesion. Downregulation of Angptl7 activated Nrf-1/HO-1 and attenuated NF-κB signaling pathway. Manifest evidence indicated the close correlation between atherosclerosis and endothelial dysfunction (Gimbrone and Garcia-Cardena 2016). The current data proved the potential of Angptl7 in endothelial dysfunction. Nevertheless, further studies are needed to investigate the mechanism of it.

Acknowledgment. Not applicable.

Foundation. Lanzhou Talent Project for Innovation and Entrepreneurship (2015-RC-12).

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Received: August 5, 2019
Final version accepted: December 15, 2019