Original Article
Silencing profilin-1 confers protection from oxLDL injury in human vascular endothelial cells

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Received November 30, 2016; Accepted January 12, 2017; Epub June 1, 2018; Published June 15, 2018

Abstract: Oxidized low density lipoprotein (oxLDL) plays a key role in the dysfunction, injury and apoptosis of vascular endothelial cells (ECs). Profilin-1, an actin-binding protein, is up-regulated during these processes of endothelial cells. The aim for this study is to investigate expression of profilin-1 in human vascular endothelial cells (HUVECs) in present of oxLDL and the protection effect by Silencing profilin-1 on HUVECs exposed to oxLDL and its mechanism. HUVECs were cultured and exposed to oxLDL (50 µg/ml) for 24 h and subsequently were treated with siRNA to inhibit gene profilin-1. Expression of protein and mRNA for profilin-1 and genes (caspase-3, Bax, Bcl-2, NF-κB and survivin) associated with apoptosis were determined using real-time PCR and western blot analysis, respectively. The cells injuries and apoptosis were examined by flow cytometry analysis. After exposed to oxLDL, the mRNA and protein levels of profilin-1 in HUVECs were significantly increased. Suppressed profilin-1 expression in oxLDL treated HUVECs by RNA-interference resulted in significant reduction and elevation of the mRNA and protein of caspase-3 and Bax, Bcl-2 and NF-κB and survivin, respectively. In addition, this is the first time that reports survivin involving oxLDL induced ECs injuries. Flow cytometry analysis showed the apoptosis ratio of oxLDL treated HUVECs significantly decreased after silencing profilin-1. These findings suggest that profilin-1 may play a key role in process of ECs injuries and apoptosis caused by oxLDL, and mechanism of its action involves various apoptosis related genes and deserves more attention.

Keywords: Profilin-1, endothelial cells, oxLDL, apoptosis

Introduction
Vascular endothelial cells (ECs) play an important role under physiological conditions, including regulations of vascular endothelial barrier function, vasodilation, antioxidant and anti-inflammation [1-3]. ECs can synthetize and release many factors simulated by mechanically and chemically, like prostacyclin, NO and bradykinin, to regulate the vasodilation and cell growth [4-6]. Plentiful investigations showed that ECs injury is the crucial role in many diseases such as systemic inflammatory response syndrome, sepsis, autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis), metabolic diseases (diabetes, obesity) and vessel related diseases (hypertension, atherosclerosis), receiving extensive attention [7-9]. Factors associated with endothelial injury include autoantibodies in autoimmune diseases, diseases (hypertension, hyperlipidemia, diabetes) and oxidative stress, and appear to be different characteristics and regulation mechanisms [10, 11].

Overproduction of reactive oxygen species (ROS) in tissues induced by variety injuries and simulations, namely oxidative stress (OS), causes the damage of antioxidation defensive system (AODS), increases protein enzyme secretion and oxidized intermediates production, reduces the capability of cleaning free radical, accumulates extensive oxygen free radical and related products and thereby disables the balance of oxidative and anti-oxidative functions [12-14]. Excessive ROS in vascular wall modify the low-density cholesterol to be oxidized low density lipoprotein (oxLDL) which promote the formation and development of various diseases through a series mechanisms such as increas-
ing vascular smooth muscle and cytotoxicity, accelerating formation of foam cells and changing fibrinolysis and coagulation [15, 16]. Previous investigations showed that low level of oxLDL can increase cell proliferation [17], whereas high level of that causes cell injuries and apoptosis in different mechanisms [18, 19]. However, one of well-known injury for ECs is recombination of actin cytoskeleton, including the dynamic process of polymerization and depolymerization of actin [20, 21]. Profilin-1, an actin binding protein with low molecular weight, exists widely in various tissues and plays an important role in ECs injuries process by regulating the polymerization and depolymerization of actin, cell proliferation and differentiation and motility, and signal transduction [22-24].

In vitro experiments demonstrated that many factors for inducing human vascular endothelial cells (HUVECs) injuries, such as oxLDL, homocysteine and cholesterol oxides, up-regulate the expression of profilin-1 in ECs and then results in the rearrangement of cytoskeleton of ECs [25, 26]. Investors proved previously that overproduction of oxygen free radical in ECs was induced after binding to oxLDL, resulted in signaling cascades and thereafter simulated protein kinase and nuclear factor (NF-κB). Subsequently, the expression of profilin-1 was increased and thereby induced reorganization and redistribution of cytoskeleton which caused pathological basis such as vasoconstriction and increased vascular permeability, and further promoted apoptosis of ECs [27, 28].

Here, we examined the role of profilin-1 in the process of oxLDL induced HUVECs injuries by determining expression of the signal transduction pathway protein NF-κB/survivin and downstream apoptosis-related protein caspase-3, Bax and Bcl-2. So as to we can evaluate whether profilin-1 was the potential therapeutic target in diseases associated with oxLDL induced ECs injuries.

Materials and methods

Antibodies and reagents

Endothelial Cell Medium (ECM) was obtained from Sciencell (USA). Trypsin and EDTA was obtained from Sigma-Aldrich Co (St. Louis, Mo.). Polyclonal antibody for Factor VIII Related Antigen is a product of USBiological (USA).

Annexin V-FITC/PI Apoptosis Detection Kit is a product of BD Pharmingen (San Diego, CA). Monoclonal antibodies for CD31 and GAPDH were purchased from GeneTex (Irvine, CA, USA). Polyclonal antibodies for cleaved caspase-3 (Rabbit IgG), Bax (Rabbit IgG), Bcl-2 (Rabbit IgG), nuclear factor κB (NFκB) and survivin are products of Cell Signaling Technology (Beverly, MA, USA). oxLDL was purchased from Luwen Biotechnologies CO., LTD. (Shanghai, China). TBARS Assay Kit is a product of Cayman (USA).

HUVECs isolation, culture and identification

HUVECs were used in this study and obtained from human umbilical cord vein as described previously [29]. The cells were cultured in complete ECM growth media (Sciencell, USA). The HUVECs were fixed using 4% paraformaldehyde for 10 min and then blocked using PBS containing 5% goat serum for 30 min. Subsequently, the cells were incubated with PBS containing mouse anti-CD31 monoclonal antibody (1:200) (cell Signaling Technology, USA) at 4°C overnight and thereafter incubated with FITC-conjugated rabbit secondary antibody (1:300) (Invitrogen, USA) at 37°C for 30 min. The HUVECs were counterstained with DAPI (Invitrogen, USA) for 5 min.

Preparation of oxLDL and cell experiments

oxLDL was obtained from Luwen Biotechnologies CO., LTD. (Shanghai, China). Oxidation of oxLDL was assessed with thiobarbituric acid-reactive substance (TBARS) value as described previously [30]. Confluent cultures of HUVECs were incubated in ECM containing oxLDL (50 µg/ml) for 24 h and were used as treated group (n=5), and the control group were incubated in ECM without adding oxLDL and used as control group (n=5). The expression of profilin-1 in oxLDL treated group and control group were examined with RT-PCR and western blot analysis.

RNA silencing profilin-1

HUVECs mentioned above were cultured in ECM containing oxLDL (50 µg/ml) for 24 h. The profilin-1 siRNA duplex synthesized in vitro by GenePharma Co., Ltd (Shanghai, China) and designed as described previously [31] are composed of sense strand (5-AGA AGGUGU CCA CGG UGG UUU-3) and antisense-strand (5-ACC
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ACC GUG GAC ACCUUCUUU-3), which was then transferred into oxLDL treated HUVECs according to the manufacturer’s instruction and used as transfection group (siRNA treated group). The control siRNA (sense strand 5'-UAG CGA CUA AAC ACA UCA AUU-3; antisense strand: 5'-UUG AUG UGU UUA GUC GCU AUU-3) designed no homology with the gene of mouse and human transfected into oxLDL treated HUVECs and was used as a mock transfection group (mock group). The third group (oxLDL treated HUVECs) was not transfected with siRNA and used as a non-transfection group (or control group). Briefly, HUVECs were transferred using 100 nM of profilin-1 siRNA for 24 h, and subsequently was cultured in growth media for another 24-72 h.

Real-time RT-PCR

Total cellular RNA from all groups was extracted using RNeasy Mini Kit (Takara Biotechnology, Dalian, China). cDNA was synthesized with a iScript cDNA synthesis kit from Bio-Rad according to the manufacturer’s instructions. The primers used were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Real-time quantitative RT-PCR was conducted using an all-in-one qPCR Mix (GeneCopoeia, Rockville, MD, USA) according to the manufacturer’s instructions. The reactions were performed in duplicate for each sample. The generation of specific PCR products was confirmed by melting curve analysis and gel electrophoresis. Data were normalized using the delta-delta CT method with respect to GAPDH as the housekeeping gene. The primers specific for profilin-1, caspase-3, Bax, Bcl-2 and NF-κB/survivin were used in RT-qPCR assays as following: profilin-1: 5'-CCGGGTGGAACGCCTACATC-3' (forward), 5'-GACGGAGGGCGAAGTGATG-3' (reverse); GAPDH: 5'-CGGGAAACTGTGGCGTGATG-3' (forward), 5'-ATGACCTTGCCCA CAGCCTT-3' (reverse); caspase-3: 5'-TGTTCCATGAAGGCAGAGCCA-3' (forward), 5'-GGACATGGTGAAGGCTGGGA-3' (reverse); BCL2: 5'-GGACATGGTGAAGGCTGGGA-3' (forward), 5'-AACTCACAGGTGGGCAAGG-3' (reverse); survivin: 5'-TACGCCTGTAATTACCACAGGAC-3' (forward), 5'-TCTC CAGAGTTTCTACCCATC-3' (reverse).

Western blot

The oxLDL treated HUVECs from profilin-1 siRNA transfection group, mock transfection group and non-transfection group were used to isolate total protein, homogenized and solubilized in ice-cold PBS containing protease inhibitors and detergent NP-40. Electrophoresis was conducted on 10% SDS-PAGE slab gel (Goodbio Technology, Wuhan, China) and the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes polyvinylidene difluoride membranes (Millipore, Billerica, MA) using Trans-blot semidry electrophoretic transfer cell (Bio-Rad, Richmond, CA). After blocking using 5% non-fat dry milk, blots were incubated with appropriate antibody (cell Signaling Technology, USA) overnight at 4°C. After washing, blots were incubated with the secondary antibody. Blots were developed with the enhanced chemiluminescence western blotting detection system ‘ECL+Plus’ (Amersham Pharmacia Biotech, Arlington Heights, IL). Quantification of proteins was obtained by analyzing the intensity of the bands using ImageJ software (NIH, USA).

HUVECs apoptosis assay

The apoptosis of oxLDL treated HUVECs collected from profilin-1 siRNA transfection group, mock transfection group and non-transfection group was investigated by flow cytometer after the HUVECs were performed with Annexin V-FITC/PI Apoptosis Detection Kit from BD Pharmingen (San Diego, CA).

Data statistics

All data are expressed as means ± SE. P < 0.05 was regarded as a statistically significant in two-tailed tests. Statistical comparisons for groups with different treatments were conducted with SPSS software (SPSS version20; IBM SPSS; USA).

Results

Identification of HUVECs

The primary endothelial cells isolated from human umbilical venous were originally found round and oval (Figure 1A), and appeared to be spindle and polygonal shape after cultured for 5-7 days (Figure 1B). The expression of vascular endothelial cells specific antigen markers CD31 (red) and Factor VIII related Antigen (green) were determined using cell immunofluorescence assay. As depicted in Figure 1C and
results showed that protein levels of profilin-1, caspase-3 and Bax in siRNA treated group were apparent lower compared to that of control and mock group (Figure 2D, 2H). However, obvious higher protein expression of Bcl-2 was observed in siRNA treated group with respect to that of the control and mock group (Figure 2H). We also determined mRNA and protein levels of the signaling pathway related gene NF-κB and survivin in profilin-1 siRNA treated group, control and mock group. Compared with siRNA treated group, there were significant lower mRNA expression of NF-κB and survivin in control and mock group (Figure 2I, 2J). Western blot assays showed that the protein level of NF-κB in control and mock group were apparent lower compared to the siRNA treated group and the significant higher expression of survivin in the siRNA treated group was achieved compared to the control and mock group (Figure 2K). All the western blot assays mentioned above were convinced with the equal value of GAPDH for each group (Figure 2H-K).

**Cell apoptosis assay**

Flow cytometry using fluorescein annexin V-FITC/PI double labeling method was performed and the results showed that protein levels of profilin-1, caspase-3 and Bax in siRNA treated group were apparent lower compared to that of control and mock group (Figure 2D, 2H). However, obvious higher protein expression of Bcl-2 was observed in siRNA treated group with respect to that of the control and mock group. There was no significant difference between control group and mock group for the expression of profilin-1, caspase-3, Bax and Bcl-2, respectively (Figure 2C, 2E-G). With regard to the protein expression, the western blot were performed and the results showed that protein levels of profilin-1, caspase-3 and Bax in siRNA treated group were apparent lower compared to that of control and mock group (Figure 2D, 2H). However, obvious higher protein expression of Bcl-2 was observed in siRNA treated group with respect to that of the control and mock group (Figure 2H). We also determined mRNA and protein levels of the signaling pathway related gene NF-κB and survivin in profilin-1 siRNA treated group, control and mock group. Compared with siRNA treated group, there were significant lower mRNA expression of NF-κB and survivin in control and mock group (Figure 2I, 2J). Western blot assays showed that the protein level of NF-κB in control and mock group were apparent lower compared to the siRNA treated group and the significant higher expression of survivin in the siRNA treated group was achieved compared to the control and mock group (Figure 2K). All the western blot assays mentioned above were convinced with the equal value of GAPDH for each group (Figure 2H-K).
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Figure 2. Silencing profilin-1 reversed the oxLDL simulated expression of caspase-3, Bax and Bcl-2 via NF-κB/survivin pathway. After exposed to oxLDL (50 µg/mL) for 24, profilin-1 level of mRNA (A) and protein (B) were significantly elevated compared to that of untreated control group. Profilin-1 silencing using siRNA was performed in cells treated with oxLDL (50 µg/mL) for 24 h (siRNA group), and its mock and control group was conducted at the same time. And the results showed that overexpression of profilin-1 mRNA and protein triggered by oxLDL were significantly decreased by silencing of profilin-1 with siRNA treatment (C, D), the levels of mRNA and proteins for caspase-3 (E, H) and Bax (F, H) were significantly decreased for the siRNA treatment group and that of Bcl-2 (G, H) was significantly elevated, the expression of mRNA and proteins for both NF-κB and survivin were increased compared to that of mock and control group (I-K). *P < 0.05 significantly different from control.

formed to detect the apoptosis of the HUVECs for the siRNA treated group, control and mock group. The ratios of late necrotic cells (B1: Annexin V-FITC+/PI+), dead cells (B3: Annexin
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al, proliferation, apoptosis and migration of cancer cells [34, 35]. However, the underlying mechanism causing up-regulation of profilin-1 in ECs induced by oxLDL is unknown. Therefore, we determined the effects of silencing profilin-1 expression by siRNA on the levels of neovascularization, cell proliferation and apoptosis associated signaling pathways such as NF-κB/survivin, and caspase-3, Bax and Bcl-2. Our findings showed that abnormal over expression of profilin-1 induced by oxLDL in control and mock group increased the levels of mRNA and protein of caspase-3 and Bax, and decreased that of Bcl-2 (*Figure 2*).

Figure 3. ECs from siRNA group, and its mock and control group, pretreated with oxLDL (50 µg/mL) for 24 h, were conducted flow cytometry analysis to determine the ratio of apoptosis. (A) The apoptosis rate of was determined in cells from control, mock and siRNA group. The proportion of late necrotic cells (B1: Annexin V-FITC+/PI+), dead cells (B3: Annexin V-FITC+/PI+) and early apoptotic cells (B4: Annexin V-FITC+/PI-) for control (B), mock (C) and siRNA (D) group were presented in the figure. *p < 0.05 significantly different from control.

V-FITC+/PI+), living cells (B2: Annexin V-FITC+/PI-) and early apoptotic cells (B4: Annexin V-FITC+/PI-) were showed in Figure 3. The proportions of late necrotic cells plus dead cells in control and mock group were significant higher compared to that of siRNA treated group. The highest ratio of living cells was achieved in siRNA treated group (*Figure 3D*).

Discussion

The present work firstly demonstrated that the expression of profilin-1 in HUVECs increased as they were exposed to high concentration oxLDL (50 µg/ml). Abnormal appearance of oxLDL on vascular endothelial cells induced the development of foam cells, inflammatory response and cell injury, which further causing the development of endothelial dysfunction related cardiovascular diseases [32]. However, the underlying mechanism of oxLDL induced Ecs injuries remains unclear. In this study, we proposed a hypothesis that over expression of profilin-1 is a key role in the mechanism of oxLDL induced ECs injuries. Our result suggested that significantly increasing of profilin-1 induced by oxLDL participates in the process of endothelial dysfunction since it was demonstrated to be an important factor during the formation of hypertension, diabetes and atherosclerosis [33]. Our suggestion was consistent with findings that profilin-1 is involved the downstream signaling transduction pathways of cardiovascular disease related vessel injuries induced by oxLDL and AGES [32]. Other investigations suggested that profilin-1 may be a key role leading rearrangement of cytoskeleton and endothelial tissue injuries induced by diabetes and atherosclerosis because profilin-1 affects the apoptosis, proliferation and differentiation of ECs by directly meditating the polymerization and depolymerization of G-actin [1, 31, 32]. In addition, profilin-1 participates development of cancers by regulating the survival, proliferation, apoptosis and migration of cancer cells [34, 35].

However, the underlying mechanism causing up-regulation of profilin-1 in ECs induced by oxLDL is unknown. Therefore, we determined the effects of silencing profilin-1 expression by siRNA on the levels of neovascularization, cell proliferation and apoptosis associated signaling pathways such as NF-κB/survivin, and caspase-3, Bax and Bcl-2. Our findings showed that abnormal over expression of profilin-1 induced by oxLDL in control and mock group increased the levels of mRNA and protein of caspase-3 and Bax, and decreased that of Bcl-2 (*Figure 2*).

Caspase family genes are associated with apoptosis and among which caspase-3 is the final executant role during apoptosis [36]. Bcl-2 family genes are the major mediators of apoptosis for mitochondrial pathway by changing the mitochondrial permeability transition and releasing cytochrome C and thereby regulating the activation of caspase-3 [36]. In addition to the levels of Bax and Bcl-2, the ratios between

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them affect the excretion of cyto C from mitochondria and subsequent signaling cascade caused by caspase-3 by changing the mitochondrial permeability transition [37]. Our results suggested that transcriptional up-regulation of caspase-3 and Bax, and down-regulation of Bcl-2 participate the apoptosis of HUVECs induced by oxLDL. Similar apoptosis showed that the ratio of hyperglycaemia induced apoptosis of ECs is positive to Bax and caspase-3, and negative to bcl-2 [38]. Romeo et al proposed that profilin-1 involves AGES and ox-LDL induced injuries in Ecs [32]. These findings suggested that AGES and ox-LDL induced injuries in Ecs probably share the same mechanism, in which profilin-1 plays a key role in the processes of cell injuries and apoptosis, and caspase-3 is the final executant of ECs apoptosis induced by oxLDL or AGES.

Our results confirmed this deduction by silencing of profilin-1 using siRNA. Down-regulation of profilin-1 attenuated HUVECs lesions induced by oxLDL, decreased the levels of Bax and caspase-3, and increased that of Bcl-2 (Figure 2). Furthermore, Zaidi AH et al revealed that profilin-1 overexpressing in cells exposed to doxorubicin, vinblastine, and benzofuran results in enhanced cell death by elevating level of p53, which confirmed by activation of caspase family, such as caspases 3, 8, 9 [19, 39]. Therefore, increasing levels of profilin-1 in cells in the presence of inducers, such as oxLDL, AGES and chemotherapeutic agents: paclitaxel and vinblastine, enhance apoptosis and cell injuries by activation of caspase-3 via elevated p53 expression.

Activation of inflammatory and apoptosis related signal transduction pathway such as AP-1, NF-kB, STAT1/3, NFAT, HIF1 and P53 are closely associated with ECs lesions induced by oxLDL [40]. NF-kB is considered an important factor involving inflammatory, cell growth, differentiation and apoptosis, which triggered by inhibiting cell apoptosis signaling, increasing abnormal proliferation and neovascularization [27]. Consequently, we attempt to explore the relationship between profilin-1 and NF-kB, and whether NF-kB involving signaling pathway in ECs injuries induced by oxLDL.

Recent investigation showed that profilin-1 overexpression induced by vinblastine and paclitaxel in cells inhibits the activation of NF-kB and its related gene by attenuating degradation of IκBα, thereby which prevents p65 nuclear translocation and reduces NF-kB DNA-binding activity [39]. Our results showed that mRNA and protein levels of NF-kB in HUVECs decreased after up-regulated profilin-1 induced by oxLDL, and increased after siRNA treatment of profilin-1 (Figure 2). These findings indicated that NF-kB is an important action in process of cell injuries and apoptosis triggered by profilin-1 overexpression, and IκBα is a key intermediate protein.

Survivin is a newly fund and strong inhibitor of apoptosis protein. In the process of apoptosis inhibition induced by survivin, caspase-3 was final signaling pathway that was inhibited [28, 41]. In president study, we firstly fund that there was significant lower levels of survivin control and mock group compared to the profilin-1 siRNA treatment group. In addition to our findings mentioned above, flow cytometry was performed to detect the apoptosis of the oxLDL treated HUVECs cultured in profilin-1 siRNA transfection, mock transfection and control (no treatment) group, and the results confirmed that the ratio of apoptosis in siRNA treated group was evident lower than that of control and mock group (Figure 3).

In conclusion, our findings suggested that down-regulation of profilin-1 by siRNA treatment can attenuate oxLDL induced apoptosis in HUVECs by mediating signaling pathways, such as inhibition the activity of caspase-3, elevation the ratio of Bax and Bcl-2, and activation of NF-kB/survivin. However, the mechanism that profilin-1 affects survivin expression is unclear and deserves further investigation.

Acknowledgements

This work was partly supported by Fuyang Science and Technology Bureau of Hangzhou, Zhejiang (No. 2015SK010).

Disclosure of conflict of interest

None.

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