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Upregulation of Nuclear Factor IA Suppresses Oxidized Low-Density Lipoprotein-Induced Endoplasmic Reticulum Stress and Apoptosis in Human Umbilical Vein Endothelial Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Endoplasmic reticulum stress (ERS) is part of the cardiovascular pathological processes, including atherosclerosis. Nuclear factor IA (NFIA) influences atherosclerosis development; however, its effects on ERS remain unknown. This study investigated the effect of NFIA on oxidized low-density lipoprotein (ox-LDL)-induced ERS and apoptosis in endothelial cells.

Material/Methods: Ox-LDL was used to induce lipotoxicity in human umbilical vein endothelial cells (HUVECs) to establish an *in vitro* oxidative injury model transfected with pcDNA3.0-NFIA. The cytotoxic response was detected using an assay to determine the release of lactate dehydrogenase (LDH). Morphological changes in cell apoptosis were detected using Hoechst 33258 staining. The proportion of apoptotic cells, releases of reactive oxygen species (ROS), and mitochondrial membrane potential ($\Delta\Psi m$) were determined using flow cytometry. The expression levels of apoptosis- and ERS-related molecules were detected through Western blotting.

Results: NFIA expression was downregulated in the *in vitro* oxidative cell-injury model. Exposure of HUVECs to ox-LDL resulted in a significant increase in apoptosis, decrease in ROS levels, and loss of $\Delta\Psi m$. Overexpression of NFIA remarkably inhibited ERS and mitochondrial-mediated apoptosis induced by ox-LDL in HUVECs by reversing the effect of ox-LDL on the expression of JNK1, p-JNK1, CHOP, Cyt C, and Bax.

Conclusions: These results demonstrated that NFIA might have beneficial effects in the prevention of ox-LDL-induced ERS and apoptosis in vascular endothelial cells. This study provided new insights into the mechanism of atherosclerosis.

MeSH Keywords: Apoptosis • Atherosclerosis • Endoplasmic Reticulum Stress • Endothelial Cells

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Background

Atherosclerosis, a unique form of arteriosclerosis, is a chronic inflammatory disease of the blood vessels and represents the most frequent and common pathological cause of cardiovascular diseases [1]. Related studies have showed that the integrity and normal function of endothelial cells play an important role in the vascular homeostasis of blood vessels and that vascular endothelial dysfunction is the initial event causing atherosclerosis [2,3]. Endothelial dysfunction is characterized by vascular endothelial cell apoptosis and is usually accompanied by vascular inflammation, changes in vascular wall morphology, and predictable cardiovascular events [4,5]. Therefore, protecting endothelial cells against this dysfunction and concurrent damages might be an effective strategy to prevent or treat atherosclerosis.

Accumulating evidence has indicated that endothelial injury and apoptosis during atherosclerosis could be induced by various risk factors such as oxidized low-density lipoprotein (ox-LDL), advanced glycation end products, and angiotensin II [6]. Among these factors, ox-LDL is a critical mediator of the initiation of endothelia injury and progression of atherosclerosis by inducing oxidative stress in endothelial cells, disrupting the antioxidant and secretory activities of vascular endothelium, and inducing endothelial apoptosis, leading to the accumulation of lipid-rich macrophages and formation of atherosclerotic plaque [7,8]. Production of reactive oxygen species (ROS) in vascular endothelial cells causes an increased level of ox-LDL [9–11].

ERS (endoplasmic reticulum stress) has been demonstrated as a new anti-atherogenic therapies according to previous reference [12]. Excessive and prolonged endoplasmic reticulum stress (ERS) induces apoptosis by enhancing the overexpression of CCAAT/enhancer-binding protein-homologous protein (CHOP) [13–15]. ERS is also involved in apoptosis by generating ROS or disturbing Ca^{2+} homeostasis in the mitochondria, thus, leading to activation of caspase-3, an initiator of caspase-dependent apoptosis [16]. Notably, a study from Tao et al. [10] demonstrated further that ox-LDL promotes the apoptosis of vascular endothelia cells largely through the protein kinase RNA-like endoplasmic reticulum kinase (PERK)/eukaryotic translation initiation factor 2α-subunit (eIF2α)/CHOP ERS pathway. Given the important role of ox-LDL in endothelial injury, it is critical that we explore the mechanisms that regulate ERS and endothelial cell apoptosis and search for potential targets to block the ox-LDL-induced endothelial injury to better prevent and treat atherosclerosis.

The nuclear factor I (NFI) family consists of transcription factors that not only affect lipid metabolism but also regulate lipid homeostasis and inflammation in macrophages, endothelial cells, and smooth muscle cells within the arterial walls [17,18]. As site-specific DNA-binding proteins, these transcription factors, including NFIA, NFIB, NFIC, and NFIX/NFID, recognize and bind the palindromic DNA sequences 5'-TTGGC(N)5GCCAA-3' and are first described as the genes required for viral replication and regulation of gene expression [19]. NFIA, a member of the NFI family, plays a main role in regulating lipid droplet formation during differentiation and participates in astrocyte differentiation in the developing central nervous system [19,20]. In addition to its role in glial development, NFIA influences atherosclerosis development by regulating the expression of genes and proteins involved in the maintenance of cholesterol homeostasis and inflammatory reactions [21]; however, the molecular mechanisms by which NFIA influences atherosclerosis development are not fully understood.

In the present study, first, we examined intracellular NFIA expression and ERS and ER apoptosis in ox-LDL-treated human umbilical vascular endothelial cells (HUVECs). Then, we conducted a gain-of-function assay to investigate the effects of NFIA on ox-LDL-induced HUVEC injury. In addition, we explored the underlying mechanism through which NFIA has a protective effect to determine whether it reduces ox-LDL-mediated ERS and regulates the ox-LDL-triggered apoptotic pathway in endothelia cells.

Material and Methods

Experimental materials

Dulbecco's modified Eagle's medium and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). Ox-LDL was purchased from Beijing Xiesheng Biotechnology (Beijing, China). The lactate dehydrogenase (LDH) assay kit was purchased from Nanjing Jiangcheng Bioengineering Institute (Jiangsu, China). The reactive oxygen species (ROS) assay kit was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). The JC-1 and Apoptosis Detection Kit was purchased from Bestbio Biotechnology (Shanghai, China). Primary antibodies against NFIA, Cyt C, Bax, JNK1, p-JNK1, and CHOP were from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture

HUVECs were obtained from the American Type Culture Collection (cat no: PCS-100-010, Manassas, VA, USA) and were cultured in endothelial cell culture medium (ScienCell, Carlsbad, CA, USA) supplemented with 5% FBS, 1% endothelial cell growth factor (ScienCell) and 100 U/mL penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator containing 5% CO_2 at 37°C.

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Cell transfection and treatment

The eukaryotic NFIA expression vector pcDNA3.0-NFIA was constructed by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). For the *in vitro* experiments, HUVEC cells were divided into the following 4 groups: (1) blank contained only RPMI 1640 medium, (2) ox-LDL contained HUVECs treated with 50 μM ox-LDL for 24 h, (3) ox-LDL + pcDNA3.0 contained HUVECs transfected with empty vector pcDNA3.0 for 48 h before adding ox-LDL, (4) ox-LDL + pcDNA3.0-NFIA contained HUVECs that were transfected with pcDNA3.0-NFIA for 48 h before adding ox-LDL. All the cell transfections were performed using Lipofectamine 2000™ (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

LDH release assay

Cytotoxicity was evaluated using an LDH assay to determine the level of LDH released from the dead cells. Briefly, HUVECs from the different groups were inoculated onto 96-well culture plates at ~5.0×10^3 cells/mL. After centrifuging at 700×g for 5 min at room temperature (RT), the supernatant was collected to measure the released LDH. A microplate reader (Sunrise, Tecan, Germany) was used to measure the optical density of each well at 450 nm. LDH activity was calculated according to the following formula:

Unit definition: 1000 mL supernatant acted with substrate for 15 min at 37°C, and 1 μmol pyruvic acid produced in the reaction was considered as 1 unit.

Hoechst 33258 staining

For the Hoechst 33258 assay, HUVECs from the different groups were seeded at 1×10^5 cells/well into a six-well plate and grown to 80% confluence, after which the cells were fixed, were washed twice with phosphate buffered saline (PBS), and were stained with 10 μg/mL Hoechst 33258 for 15 min according to the manufacturer's instructions (Beyotime, Haimen, China). Cellular morphological changes, including nuclear condensation and fragmentation, were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Detection of apoptosis by flow cytometry

Cell apoptosis was determined using flow cytometry and double fluorescence staining with annexin V/propidium iodide (PI). In brief, before staining, HUVECs from the different groups were seeded into 6-cm culture dishes at 2×10^5 cells/well, washed with cold PBS, and suspended using 200 μL binding buffer. After staining, apoptosis was detected using a flow cytometer (BD Pharmingen, San Diego, CA, USA).

Measuring ROS

Intracellular ROS was measured using the non-fluorescent probe 2',7',-dichlorofluorescein diacetate (DCFH-DA) according to the manufacturer's instructions. To analyze ROS generation, HUVECs from the different groups were seeded into six-well culture dishes at 1×10^5 cells/well and were cultured overnight. Then, the cells were washed 3 times with PBS and were incubated with 10 μM DCFH-DA for 20 min at 37°C. The fluorescence intensity of the ROS probes was detected using flow cytometric analysis. The amount of ROS was calculated by analyzing the mean fluorescence intensity from 3 random fields using Image J v. 1.44 (*https://imagej.nih.gov/ij/*).

Measuring

The changes in mitochondrial membrane potential ($\Delta\Psi m$) were detected using a JC-1 Detection Kit according to the manufacturer's instructions. Briefly, HUVECs from the different groups were collected and grown in the 24-well plates at 1×10^5 cells/well. After washing twice with PBS and centrifuging, the cells were resuspended in 500 μL incubation buffer containing 1 μL JC-1 at 37°C for 15 min. After centrifuging the cells at 550×g for 5 min at RT, the cells were resuspended in 1x incubation buffer, after which flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was used to determine $\Delta\Psi m$.

Western blotting analysis

HUVECs from the different groups were rinsed twice with ice-cold PBS and lysed in lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich). The solution was centrifuged at 2000×g for 15 min at 4°C and the supernatant collected for protein quantification using a bicinchoninic acid (BCA) kit (Beyotime, Haimen, China). Approximately 40-μg protein samples were separated on 12% sodium dodecylsulfate (SDS)-polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at RT, the membranes were incubated with primary antibodies against NFIA, cytochrome c (Cyt C), Bax, JNK1, p-JNK1, CHOP, and GAPDH. Then, the membrane was washed 3 times with TBST and was incubated with an HRP-conjugated anti-rabbit immunoglobulin (Ig)G antibody as the secondary antibody (Cell Signaling Technology, Inc., Danvers, MA, USA). The protein signal was visualized using enhanced chemiluminescence (Pierce, Rockford, IL, USA). GAPDH served as the internal control for total proteins.

Statistical analyses

SPSS v 18.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. All quantitative data were expressed as the mean

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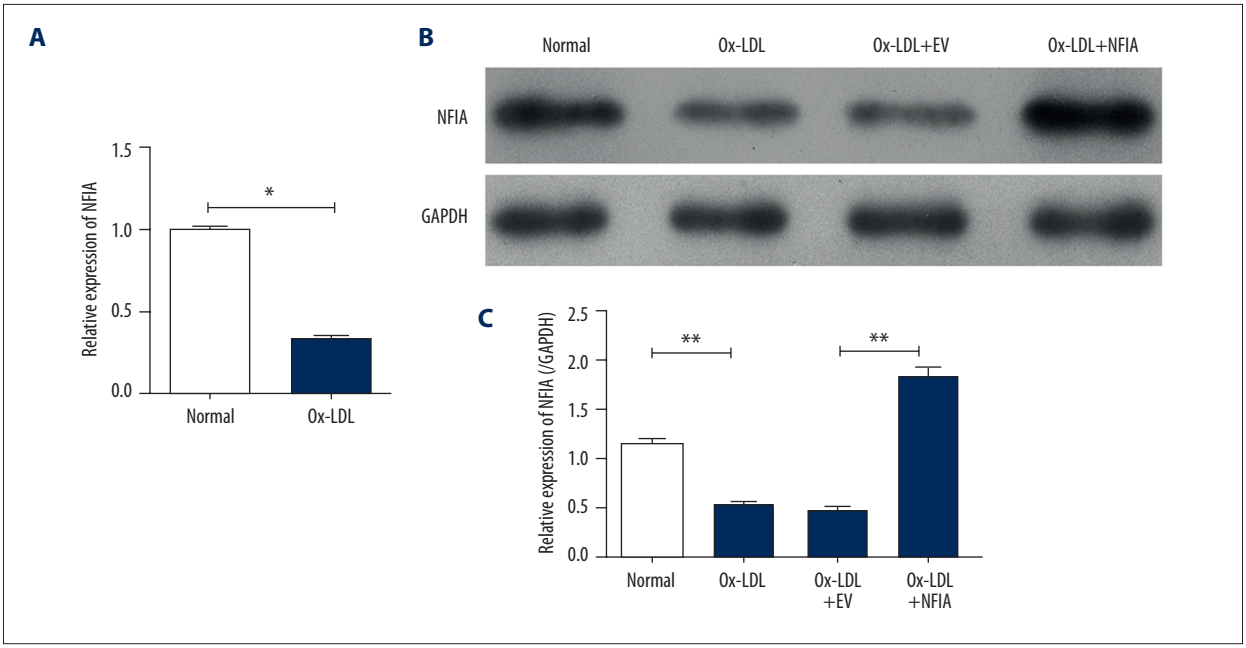

Figure 1. Expression of NFIA in ox-LDL-treated vascular endothelial cells. (**A**) expression of NFIA mRNA in ox-LDL-treated vascular endothelial cell injury. (**B, C**) Western blot analysis of NFIA expression in HUVECs. GAPDH was used as an internal control. ** $p<0.01$.

±SD from at least 3 independent experiments. One-way analysis of variance was used to evaluate differences among the groups. Differences with a $p<.05$ were considered statistically significant.

Results

NFIA alleviated the loss of ROS and ΔΨm in vascular endothelial cell injury

The expression of NFIA, a new AS modifier gene, was first determined in HUVECs and the results showed that it was obviously downregulated in the ox-LDL group compared with that in the blank group at the mRNA level (Figure 1A). Subsequently, the expression of NFIA was overexpressed by transfecting with the pcDNA3.0-NFIA plasmid in HUVECs from the ox-LDL group (ox-LDL+EV), as demonstrated by Western blot analysis (Figure 1B, 1C).

ROS production can serve as a signal to trigger apoptosis in the transduction pathway. As Figure 2A and 2B show, the intracellular ROS levels in HUVECs from blank and ox-LDL groups were 7.50±1.05% and 66.90±1.72%, respectively ($p<.01$). In contrast, that in NFIA-overexpressing HUVECs was 16.60±1.50%, which was significantly lower than that in the empty vector group (75.30±3.66%, $p<.001$). These results indicate that NFIA overexpression significantly inhibits the elevated intracellular ROS levels induced by ox-LDL in HUVECs.

Furthermore, the effects of NFIA on mitochondrial-mediated apoptosis were investigated in HUVECs by determining ΔΨm. As Figure 2C shows, HUVECs were treated with ox-LDL and then stained with JC-1 before being subjected to flow cytometry. The results of the analysis showed that ox-LDL treatment resulted in the loss of ΔΨm compared with that in the blank group (Figure 2C, 2D, $p<.01$); however, NFIA overexpression significantly reversed the decrease in ΔΨm in ox-LDL-induced HUVECs.

Upregulation of NFIA reduced apoptotic cells and LDH activity induced by ox-LDL in HUVECs through the JNK1 pathway

As Figure 3A and 3B show, exposure of HUVECs to ox-LDL resulted in a significant increase in the rate of apoptosis from 12.30±1.25% to 34.60±2.72% ($p<.01$); however, transfection with ox-LDL + pcDNA3.0-NFIA led to suppression of ox-LDL-induced apoptosis in HUVECs ($p<.01$). In addition, Hoechst 33258 staining that was used to observe apoptotic morphology (Figure 3C, 3D) showed that HUVECs in the ox-LDL group exhibited obvious apoptotic morphology, including increased condensed chromatin and unclear fragmentation. Consistently, NFIA overexpression effectively suppressed the pro-apoptotic effects of ox-LDL in HUVECs.

Cytotoxicity in each group was detected using an assay of LDH releases. As Figure 3E shows, LDH activity was significantly increased from 43.34±2.24 U/L in the control group to 104.25±8.72

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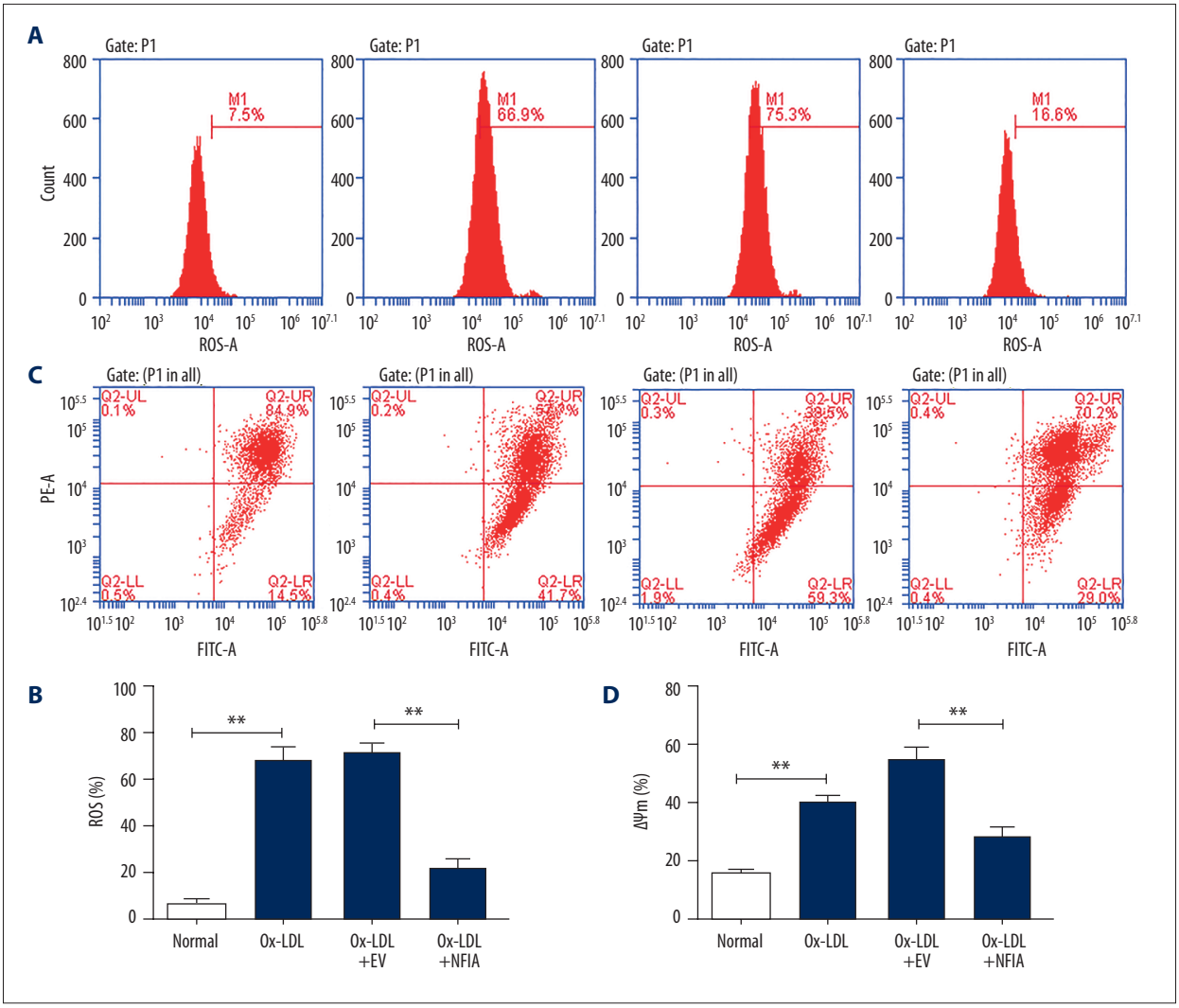

Figure 2. Effects of NFIA on vascular endothelial cell injury. (**A**) The ROS levels were measured using DCFH-DA and flow cytometry. (**B**) Quantified results of ROS generation. Data are shown as the means ±SD of 3 independent experiments. (**C**) The ΔΨm of HUVECs was stained with JC-1 and then analyzed by flow cytometry. (**D**) Quantified results of ΔΨm. Data are shown as the means ±SD of 3 independent experiments. ** $p<0.01$.

U/L in the ox-LDL group in HUVECs ($p<.001$). The results showed that NFIA overexpression decreased the release of LDH to 61.47% compared with that in ox-LDL+pcDNA3.0 group ($p<.01$).

To explore the direct effect of NFIA on ERS and mitochondrial-mediated apoptosis, ERS (JNK1, p-JNK1, and CHOP) and apoptotic marker proteins (Cyt C and Bax) were examined in HUVECs. As Figure 3F–3H show, ox-LDL treatment greatly increased the protein levels of JNK1, p-JNK1, CHOP, Cyt C, and Bax compared with those in the blank group; however, transfection with pcDNA3.0-NFIA before ox-LDL treatment obviously reversed the effect of ox-LDL on the expression of JNK1, p-JNK1, CHOP, Cyt C, and Bax. These results demonstrated further that NFIA could directly inhibit ERS and mitochondrial-mediated apoptosis induced by ox-LDL in HUVECs.

Discussion

Atherosclerosis is one of the most common of all cardiovascular diseases. ERS participates in the pathogenesis of atherosclerosis, which is characterized by cell apoptosis, increased production of ROS, and depolarization of the mitochondrial membrane potential [22,23]. A previous study has shown that antioxidants could protect the endothelial cells against ERS-induced apoptosis by directly decreasing ROS generation [24]. NFIA, a member of the NFI family, provides protection in atherosclerosis development by regulating the expression of genes and proteins involved in the maintenance of cholesterol homeostasis and inflammatory reactions [24]. In our study, we found that upregulation of NFIA inhibited the effects of ox-LDL (an ERS inducer) to increase cell viability and decrease apoptosis in HUVECs.

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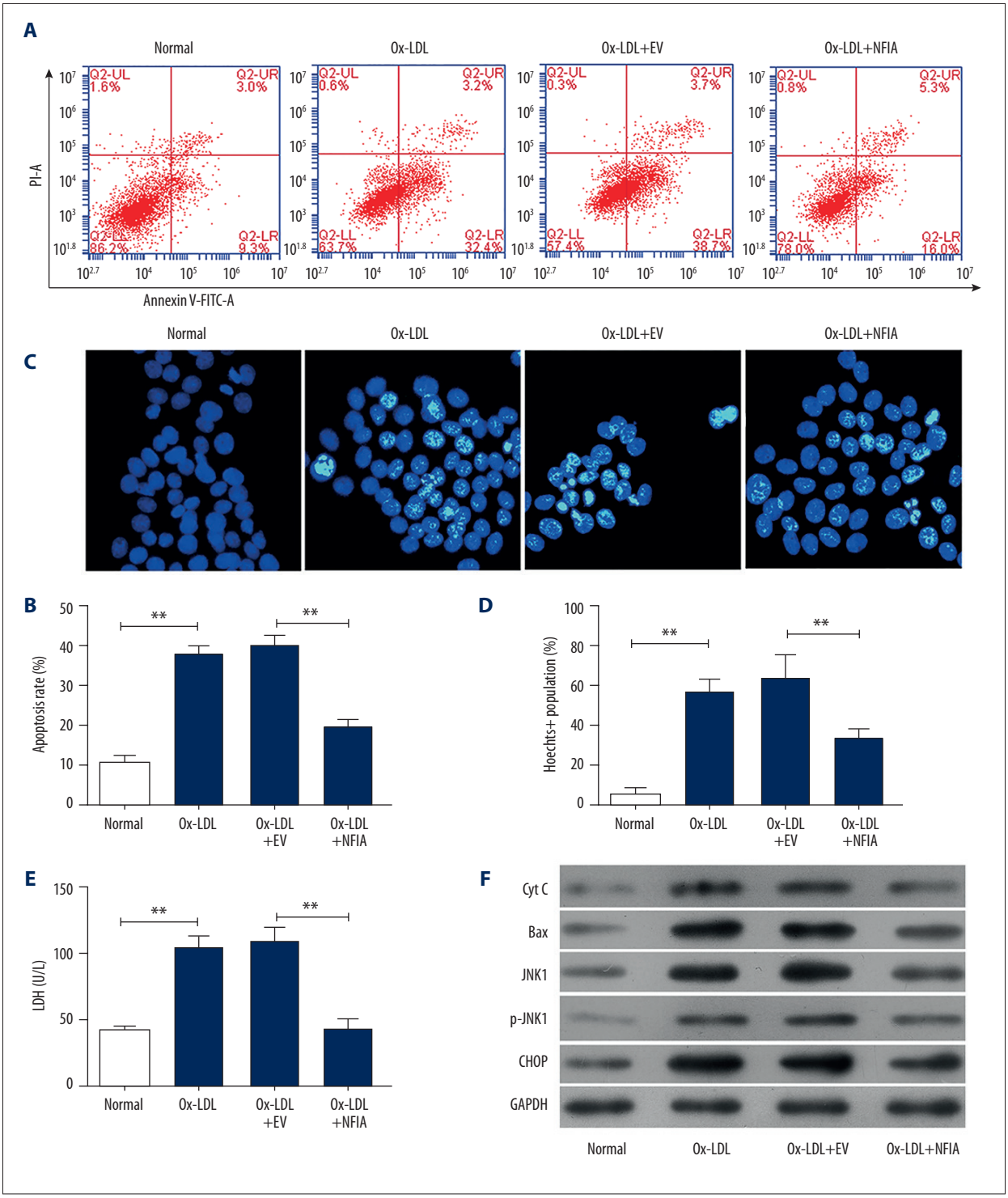
It is well accepted that ox-LDL is a factor in the initiation and progression of atherosclerosis; therefore, we speculated that abnormal expression of NFIA might be associated with atherosclerosis development, which must be demonstrated further in future studies.

Next, we investigated the mechanisms by which NFIA protects HUVECs. The results showed that upregulation of NFIA alleviated vascular endothelial cell injury by decreasing LDH level and apoptosis in HUVECs by downregulating pro-apoptotic marker proteins (Cyt C and Bax). One related study indicated that cell proliferation defects and aberrant apoptosis



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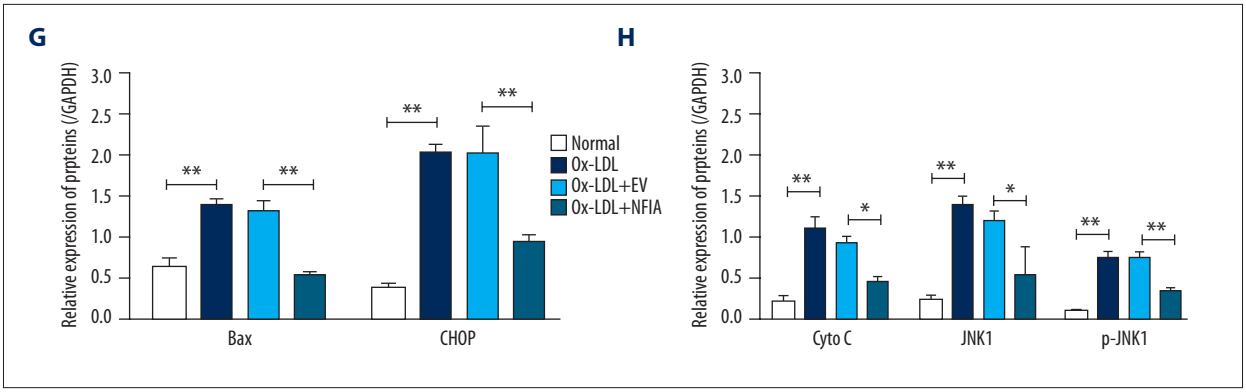

Figure 3. NFIA reduced apoptotic cells and LDH activity induced by ox-LDL in HUVECs through JNK1 pathway. (**A, B**) Cell apoptosis was measured by Annexin V/PI staining (flow cytometric analyses). Data are shown as the means ±SD of 3 independent experiments. (**C, D**) Hoechst 33258 staining was performed to visualize the extent of apoptotic cells. Condensed or fragmented nuclei were considered as apoptotic cells (magnification: 200×). (**E**) The cell injury in each group was detected via LDH release assay. Data are shown as the means ±SD of 3 independent experiments. (**F–H**) ERS (JNK1, p-JNK1 and CHOP) and apoptotic marker proteins (Cyt C and Bax) were detected in HUVECs using Western blot analysis. GAPDH was used as an internal control. ** $p<0.01$.

are key characteristics of the type of endothelial dysfunction that is involved in atherosclerosis progression [25]. Elevated levels of ox-LDL have been demonstrated to inhibit the proliferation of endothelial cells by suppressing the expression of basic fibroblast growth factor, which is essential for endothelial proliferation [26]. Recent observations have also found that NFIA significantly stimulated key genes involved in the mediation of cholesterol transport across cellular membranes and the binding and uptake of ox-LDL in macrophages [27,28]. The expression of NFIA was significantly upregulated in the anti-inflammatory effects of dihydrocapsaicin, which might be a key anti-inflammatory gene for decreasing atherosclerosis [29]. Moreover, enhancement of NFIA expression could decrease levels of lipopolysaccharide-stimulated proinflammatory cytokines, including interleukin (IL)-6 and tumor necrosis factor-α, in mice fed a high cholesterol diet [21]. From this evidence, we speculated that upregulation of NFIA could alleviate the vascular endothelial cell injury induced by ox-LDL by suppressing inflammation-related apoptosis.

In addition to apoptosis, ERS induced by ox-LDL is another key factor in the initiation and progression of atherosclerosis [30]. In this work, we provided evidence that ox-LDL treatment resulted in an effective inducer of irreversible ERS in endothelial cells, as revealed by the enhanced expressions of p-JNK1 and CHOP, which could be partially alleviated by NFIA overexpression. JNK is a stress-activated protein kinase of the MAPK family and plays vital roles in apoptosis [31]. CHOP is considered to be a critical factor in triggering apoptosis in response to ERS [32]. An additional study showed that ERS is also involved in apoptosis by generating endogenous ROS [33]. Excessive accumulation of ROS could lead to oxidative damage to lipids, proteins, and DNA by affecting MAPK signal transduction [34,35]. We also found that NFIA overexpression significantly reversed the decrease in $\Delta\Psi m$ in ox-LDL-induced HUVECs. The loss of $\Delta\Psi m$ is linked to the mitochondrial-mediated apoptotic pathway and is indicative of mitochondrial dysfunction, which could be modulated by the expressions of the Bcl-2 family protein and Cyt C [36]. These results provide an explanation of the mechanism initiated for the protective effect of NFIA on ox-LDL-induced ERS and endothelial apoptosis observed in this study.

Conclusions

Our study demonstrated that NFIA exerts a protective effect on endothelial cells by inhibiting ox-LDL-induced ER stress and apoptosis in HUVECs. This is the first study to verify that aberrant NFIA expression can mediate the survival of endothelial cells and provides new insights into the molecular mechanisms underlying endothelial injury and the pathogenesis of atherosclerosis.

Conflict of interest

None.

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