Notoginsenoside R1 upregulates miR-221-3p expression to alleviate ox-LDL-induced apoptosis, inflammation, and oxidative stress by inhibiting the TLR4/NF-κB pathway in HUVECs

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Abstract

Atherosclerosis (AS) is a common vascular disease, which can cause apoptosis of vascular endothelial cells. Notoginsenoside R1 (NGR1) is considered an anti-AS drug. MicroRNAs (miRNAs) are believed to play a vital role in cell apoptosis and angiogenesis. This study aimed to explore the mechanism of NGR1 for treating AS through miRNAs. Flow cytometry was used to detect the apoptosis rate. The levels of inflammatory cytokines interleukin (IL)-6 and IL-1β were detected using ELISA. Reactive oxygen species (ROS) and malondialdehyde (MDA) levels were measured using corresponding assay kits. Quantitative real-time polymerase chain reaction (qRT-PCR) assay was performed to detect miR-221-3p expression. Dual-luciferase reporter and RNA immunoprecipitation assays were carried out to examine the relationship between miR-221-3p and toll-like receptors 4 (TLR4). Also, western blot analysis was performed to determine the levels of TLR4 and nuclear factor kappa B (NF-κB) signaling pathway-related proteins. Oxidized low-density lipoprotein (ox-LDL) induced human umbilical vein endothelial cells (HUVECs) apoptosis, inflammation, and oxidative stress. NGR1 alleviated the negative effect of ox-LDL through promoting the expression of miR-221-3p in HUVECs. TLR4 was a target of miR-221-3p, and its overexpression could reverse the inhibition effects of miR-221-3p on apoptosis, inflammation, and oxidative stress. NGR1 improved miR-221-3p expression to inhibit the activation of the TLR4/NF-κB pathway in ox-LDL-treated HUVECs. NGR1 decreased ox-LDL-induced HUVECs apoptosis, inflammation, and oxidative stress through increasing miR-221-3p expression, thereby inhibiting the activation of the TLR4/NF-κB pathway. This study of the mechanism of NGR1 provided a more theoretical basis for the treatment of AS.

Key words: Atherosclerosis; Apoptosis; Inflammation; Oxidative stress

Introduction

Atherosclerosis (AS) is a chronic vascular disease characterized by chronic inflammation of the artery wall, which is a major health problem of the elderly worldwide (1). Oxidative stress and inflammation contribute to AS development. Vascular endothelial cell (EC) apoptosis is considered the initiation factor of the occurrence, development, and pathogenesis of AS (2). Oxidized low-density lipoprotein (ox-LDL) is a common factor in the establishment of experimental models of AS, which can induce EC apoptosis and act as an essential risk factor for the formation of AS (3–5). Therefore, inhibiting ox-LDL-induced EC apoptosis has been a new approach to treat AS.

Previous studies have shown that ox-LDL could be used as a stimulator to induce apoptosis in an *in vitro* model (6,7).

Panax notoginseng (PN) is a type of Chinese herbal medicine whose main active ingredient is panax notoginseng saponins (PNS). Studies have shown that PN has a good regulating effect on the blood and cardiovascular system (8,9). Notoginsenoside R1 (NGR1) is one of the main constituents of PNS, which has anti-inflammatory, anti-oxidative, and anti-apoptosis effects (10,11). It has been reported that NGR1, as an anti-AS drug, is involved in regulating inflammation, oxidative stress, lipid metabolism, and microRNAs (miRNAs) expression (12).

However, the functions and possible potential mechanisms of NGR1 on AS remain to be determined.

miRNAs are small non-coding RNAs of ~ 22 nucleotides in length, which are involved in cell proliferation, differentiation, invasion, apoptosis, and angiogenesis through translation, inhibition, or mRNA degradation (13,14). Studies revealed that miRNAs are highly expressed in the cardiovascular system (15). Wu et al. (16) suggested that exogenous cervical squamous cell carcinoma (CSCC)-derived miR-221-3p is transferred into human umbilical vein endothelial cells (HUVECs) and directly induces angiogenesis. Importantly, it has been well-documented that miR-221-3p is upregulated in AS and that it participates in the development of AS (17). However, the mechanism by which miR-221-3p regulates AS remains to be further studied.

Toll-like receptors (TLRs) are members of the pattern recognition receptor family and take part in inflammatory responses through activating the nuclear factor kappa B (NF-κB) signaling pathway (18,19). Early studies identified that TLRs and other critical components of the innate immune system play a critical role in the development of AS (20). Moreover, studies have shown that knockdown of TLR4 reduces the formation of AS plaque (21,22). In the present study, we determined the effect of NGR1 on ox-LDL-induced HUVECs by detecting relevant indicators of apoptosis, inflammation, and oxidative stress, and confirmed the mechanism of NGR1 through experimental verification. The discovery of the miR-221-3p/TLR4/NF-κB pathway will provide new ideas for the study of AS treatment.

Material and Methods

Cell culture

HUVECs were purchased from American Type Culture Collection (ATCC, USA) and cultured in RPMI-1640 medium (Gibco, USA), 10% fetal bovine serum (FBS; Gibco), and 100 U/mL penicillin/streptomycin (Gibco) at 37°C in 5% CO_2 incubator.

Cell treatment and transfection

NGR1 was purchased from Azelasi Biotechnology (China) and diluted according to the manufacturer's instructions. After treatment with 30 μM NGR1 for 24 h, HUVECs were treated with 50 mg/L ox-LDL (Bioss, China) for 24 h in serum-free medium. miR-221-3p mimic and inhibitor (miR-221-3p and in-miR-221-3p) or their negative controls (miR-NC and in-miR-NC), TLR4 overexpression plasmid (TLR4), and its negative control (pcDNA) were purchased from GenePharma (China). Lipofectamine 2000 (Invitrogen, USA) was used to transfect these plasmids into HUVECs. After transfection for 24 h, HUVECs were treated with ox-LDL or NGR1.

Assessment of apoptosis

HUVECs were digested by 0.25% trypsin (Gibco) and collected into 10 mL centrifuge tubes after treatment and transfection. Annexin V-FITC Apoptosis Detection kit (Beyotime, China) was used to detect cell apoptosis. After centrifugation (5000 g, 10 min, 4°C), HUVECs were re-suspended with 200 μL Annexin V-FITC binding solution and incubated with 10 μL propidium iodide (PI) staining solution for 10–20 min in the dark. FITC fluorescence was detected using a flow cytometer (Beckman Coulter, USA).

Enzyme-linked immunosorbent assay (ELISA)

HUVECs were seeded into 6-well plates. After the cells were treated and transfected, the levels of inflammatory cytokines interleukin (IL)-6 and IL-1β were detected using ELISA kits (R&D Systems, USA) according to the manufacturer's instructions.

Reactive oxygen species (ROS) and malondialdehyde (MDA) measurement

ROS Assay kit (Beyotime) was used to detect the ROS level. After treatment and transfection, HUVECs were incubated with 10 μM DCFH-DA for 20 min. After washing with serum-free medium, the absorbance of HUVECs was detected using the enzyme-plate meter (Bio-Rad, USA). Lipid Peroxidation MDA Assay kit (Beyotime) was used to detect the MDA level. HUVECs were lysed with RIPA lysis buffer (Beyotime) and incubated with 200 μL MDA detection solution for 15 min. The supernatant was collected and added into 96-well plates to detect the absorbance using the enzyme-plate meter (Bio-Rad).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Takara, China). Prime Script™ RT reagent kit (Takara) was used to synthesize cDNA. qRT-PCR was performed using SYBR® Premix Dimer Eraser kit (Takara) on Step One Plus instrument (Life Technologies, USA). GAPDH and U6 small nuclear RNA (U6-snRNA) were used as internal controls. Data were calculated using $2^{-\Delta\Delta Ct}$ methods. The primer sequences used were: miR-221-3p, forward, 5′-CCCAG CATTTCTGACTGTTG-3′, reverse 5′-AACGCTTCACGAA TTTGCGT-3′; U6, forward, 5′-GTAGATACTGCAGTACG-3′, reverse 5′-ATCGCATGACGTACCTGAGC-3′; TLR4, forward, 5′-AACCACCTCCACGCAGGGCT-3′, reverse, 5′-TG ATGTCTGCCTCGCGCCTG-3′; GAPDH, forward, 5′-AGC CACATCGCTCAGACAC-3′, reverse, 5′-GCCCAATACGAC CAAATCC-3′.

Western blot analysis

HUVECs were lysed with RIPA lysis buffer (Beyotime) and quantified with BCA protein assay kit (Beyotime). The same amount of protein was separated by SDS-PAGE (Beyotime) and transferred onto polyvinylidene difluoride membranes (Millipore, USA). Then, the membranes were blocked with nonfat milk for 1 h and incubated with primary antibodies TLR4 (1:1,000, ab22048, Abcam, USA), p65

(1:2,000, ab32536, Abcam), p-p65 (1:2,000, ab86299, Abcam), IκBα (1:1,000, ab7217, Abcam), p-IκBα (1:1,000, ab133462, Abcam), or β-actin (1:1,000, ab8226, Abcam) at 4°C overnight. After incubation with HRP-conjugated secondary antibodies (1:1,000, Santa Cruz Biotechnology, USA) for 1 h, the membranes were washed with TBST buffer and treated with chemiluminescence. Image Reader LAS-500 (Fujifilm, Japan) was used to detect the protein signals.

Dual-luciferase reporter assay

TLR4 3′UTR fragments containing the predicted binding sites and mutant binding sites of miR-221-3p were synthesized and cloned into the psiCHECK-2 plasmids (Promega, USA) to generate TLR4 3′UTR-WT and TLR4 3′UTR-MUT, respectively. Lipofectamine 2000 (Invitrogen) was used to co-transfect the above reporter plasmids with miR-221-3p mimic or miR-NC into HUVECs. The Firefly and Renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega).

RNA immunoprecipitation (RIP) assay

RIP assay was applied to confirm the direct binding relationship between miR-221-3p and TLR4 using the Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore) according to the manufacturer's protocol. HUVECs were transfected with miR-221-3p for 48 h and then incubated with an anti-Ago2 antibody or IgG antibody. QRT-PCR was performed to detect the enrichment of TLR4.

Statistical analysis

All results are reported as means ± SD from three independent experiments. Statistical differences were determined by one-way analysis of variance (ANOVA). SPSS 19.0 software (SPSS, USA) was used in this procedure. P less than 0.05 was considered as significant results.

Results

NGR1 alleviated ox-LDL-induced apoptosis, inflammation, and oxidative stress in HUVECs

Flow cytometry results showed that ox-LDL promoted cell apoptosis, while NGR1 alleviated ox-LDL-induced apoptosis (Figure 1A). ELISA demonstrated that ox-LDL induced inflammation cytokines, including IL-6 and IL-1β, whereas NGR1 inhibited their levels (Figure 1B and C). Also, ox-LDL dramatically increased ROS and MDA levels, while the ox-LDL + NGR1 group decreased ROS and MDA levels (Figure 1D and E). These results suggested that NGR1 significantly inhibited inflammation and oxidative stress in HUVECs. Interestingly, we found that ox-LDL significantly inhibited the expression of miR-221-3p and promoted the protein level of TLR4, while NGR1 increased miR-221-3p expression and reduced TLR4 protein level (Figure 1F and G), suggesting that miR-221-3p and TLR4

may be involved in the regulation of HUVECs by NGR1 and ox-LDL.

NGR1 participated in the cellular response by promoting the expression of miR-221-3p in HUVECs

QRT-PCR results indicated that overexpression of miR-221-3p could enhance the promotion effect of NGR1 on the expression of miR-221-3p, suggesting that both of them had a synergistic effect (Supplementary Figure S1A). Flow cytometry results revealed that combined treatment with miR-221-3p mimic and NGR1 enhanced the inhibition in ox-LDL-induced cell apoptosis (Figure 2A). Similarly, through detecting the levels of IL-6, IL-1β, ROS, and MDA, we found that miR-221-3p inhibited the ox-LDL-induced inflammatory response and oxidative stress, and the inhibitory effects were more obvious when it acted together with NGR1 (Figure 2B–E). These results suggested that NGR1 increased miR-221-3p expression to alleviate ox-LDL-induced cellular response in HUVECs.

TLR4 was a target of miR-221-3p

The finding that the expression trend of miR-221-3p was contrary to that of TLF4 (Figure 1F and G), combined with bioinformatics prediction, indicated that TLR4 3′UTR had a complementary sequence with miR-221-3p (Figure 3A). To confirm the presence of direct interaction between miR-221-3p and TLR4, we performed the Dual-Luciferase Reporter assay. Ectopic expression of miR-221-3p effectively reduced the luciferase activity of TLR4 3′UTR-WT, while it did not affect TLR4 3′UTR-MUT (Figure 3B). RIP assay results showed that the introduction of miR-221-3p mimic resulted in the substantial enrichment of TLR4 in RIP-Ago2 immunoprecipitation complex compared with RIP-IgG (Figure 3C). We also examined the effect of miR-221-3p expression on the TLR4 level. Through the detection of the miR-221-3p expression, we confirmed the efficacy of miR-221-3p mimic and inhibitor (Supplementary Figure S1B). Western blot analysis indicated that miR-221-3p overexpression inhibited the protein level of TLR4, while its inhibition promoted TLR4 protein level (Figure 3D). These results suggested that miR-221-3p targeted TLR4 in HUVECs.

TLR4 overexpression reversed the suppression effects of miR-221-3p on the ox-LDL-induced cellular response in HUVECs

To assess whether miR-221-3p was involved in the cellular response by targeting TLR4, we co-transfected miR-221-3p mimic and TLR4 overexpression plasmid into ox-LDL-stimulated HUVECs. The protein detection results of TLR4 indicated that the transfection efficiency of TLR4 overexpression plasmid was excellent (Supplementary Figure S1C). Flow cytometry results showed that overexpression of miR-221-3p inhibited cell apoptosis, while TLR4 reversed the inhibition effects of miR-221-3p (Figure 4A). Meanwhile, the levels of IL-6 and IL-1β or
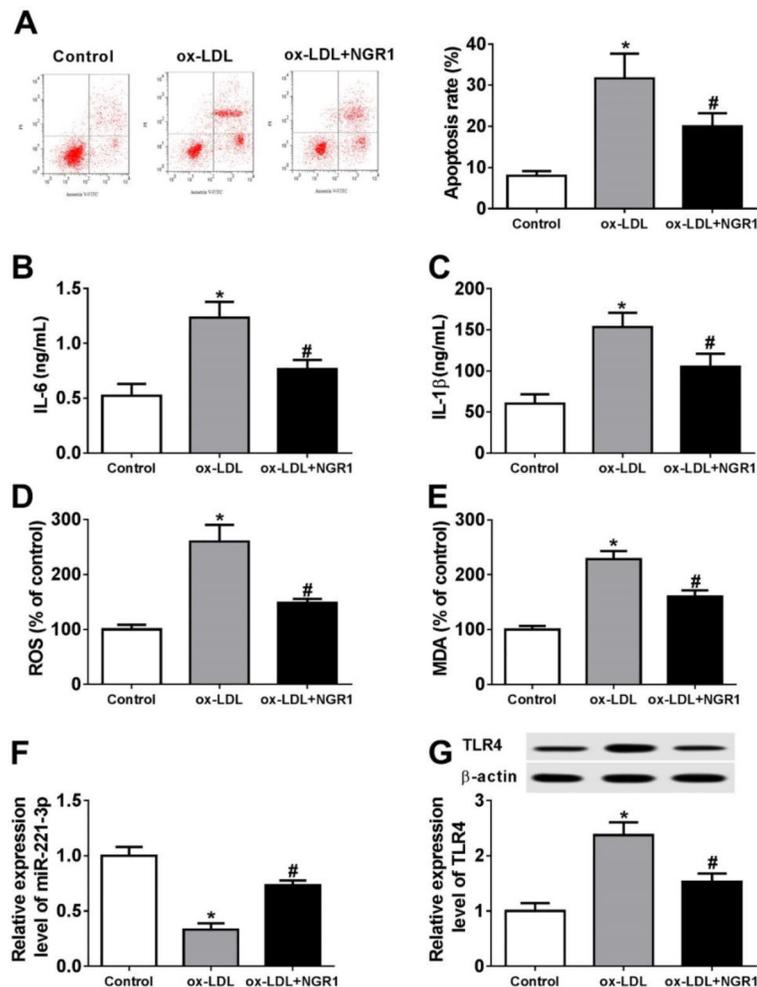
Figure 1. NGR1 alleviated oxidized low-density lipoprotein (ox-LDL)-induced apoptosis, inflammation, and oxidative stress in induced human umbilical vein endothelial cells (HUVECs). HUVECs were pretreated with or without 30 μM NGR1 for 24 h, followed by exposure to 50 mg/L ox-LDL. **A**, Flow cytometry was performed to evaluate cell apoptosis in HUVECs. **B** and **C**, ELISA assay was employed to examine the levels of inflammatory cytokines interleukin (IL)-6 and IL-1β. **D** and **E**, The levels of oxidative stress markers reactive oxygen species (ROS) and malondialdehyde (MDA) were determined by assay kits. **F**, MiR-221-3p expression was measured by qRT-PCR. **G**, TLR4 protein level was detected using western blot analysis. Data are reported as means±SD from three independent experiments. *$P < 0.05$ compared to control; #$P < 0.05$ compared to ox-LDL (ANOVA).

ROS and MDA detection showed that miR-221-3p blocked inflammation and oxidative stress, while TLR4 had the opposite effects (Figure 4B–E). The results indicated that miR-221-3p reduced the TLR4 level to improve ox-LDL-induced HUVECs apoptosis, inflammation, and oxidative stress.

NGR1 inactivated the TLR4/NF-κB pathway in ox-LDL-treated HUVECs through miR-221-3p

We further analyzed the effects of NGR1 and miR-221-3p on the TLR4/NF-κB pathway in ox-LDL-treated HUVECs. As shown in Figure 5A, the ratios of p-p65/p65 and p-IκBα/IκBα were significantly higher in ox-LDL-treated HUVECs compared with control. However, NGR1 and miR-221-3p treatment notably hindered the upregulation of p-p65 and p-IκBα induced by ox-LDL-treated HUVECs, suggesting that NGR1 and miR-221-3p inhibited the activation of the TLR4/NF-κB pathway in ox-LDL-treated HUVECs. Also, overexpression of TLF4 significantly promoted the ratios of p-p65/ p65 and p-IκBα/ IκBα in ox-LDL-treated HUVECs (Figure 5B). Collectively, these data suggested that NGR1 regulated the TLR4/NF-κB pathway through miR-221-3p.

Discussion

The development of AS is a complex process (23) and, at present, the exact mechanism of AS is still unclear.
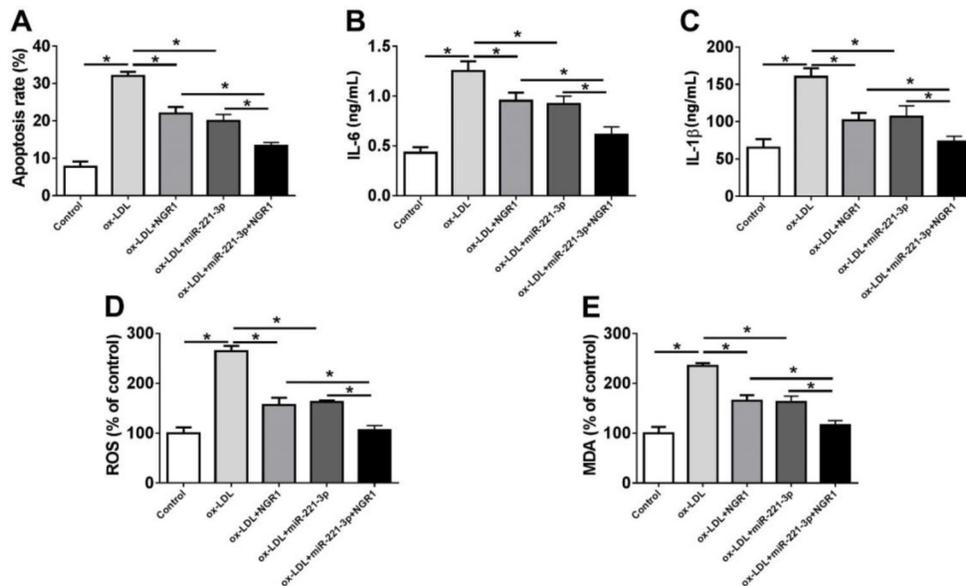
Figure 2. NGR1 participated in the cellular response by promoting the expression of miR-221-3p in induced human umbilical vein endothelial cells (HUVECs). HUVECs were pretreated with miR-221-3p mimic or NGR1, followed by stimulation with 50 mg/L oxidized low-density lipoprotein (ox-LDL). **A**, Flow cytometry was performed to evaluate cell apoptosis in HUVECs. **B** and **C**, The levels of inflammatory cytokines interleukin (IL)-6 and IL-1β were determined by ELISA. **D** and **E**, Reactive oxygen species (ROS) and malondialdehyde (MDA) levels were determined by assay kits. Data are reported as means ± SD from three independent experiments. *P < 0.05 (ANOVA).
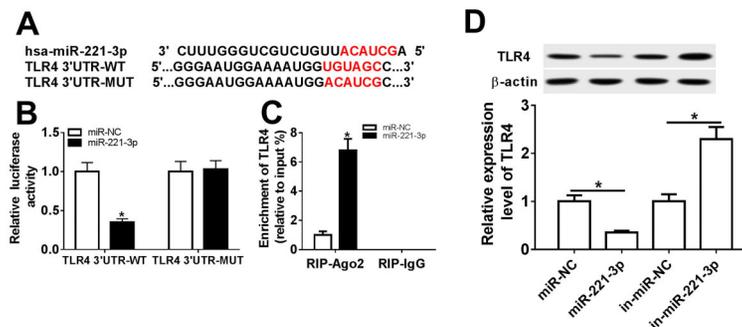

Figure 3. TLR4 was a target of miR-221-3p. **A**, The binding sites and mutant binding sites between miR-221-3p and TLR4 are shown. **B**, The luciferase activities of TLR4 3′UTR-WT/MUT (wild type/mutated) were determined by Dual-Luciferase Reporter assay. **C**, RNA immunoprecipitation assay was performed to detect the enrichment of TLR4. **D**, TLR4 protein level was tested using western blot analysis. Data are reported as means ± SD from three independent experiments. *P < 0.05 (ANOVA).

Recently, miRNAs have attracted more attention due to their critical regulatory roles in the progression of AS (24,25). In this study, we demonstrated that NGR1 had anti-apoptosis, anti-inflammatory, and anti-oxidative stress effects on ox-LDL-treated HUVECs through the miR-221-3p/TLR4/NF-κB pathway. Therefore, increasing the level of miR-221-3p might be a new strategy for the prevention and treatment of AS.

Many studies have shown that ox-LDL induced oxidative stress by activating ROS and MDA, and stimulated inflammation by releasing inflammatory cytokines (26,27). Therefore, the detection of ROS, MDA, and inflammatory cytokines levels were an effective method to measure the influence of ox-LDL on cells. In our study, we found that ox-LDL induced oxidative stress and inflammatory response of HUVECs, and NGR1 alleviated these effects. We also revealed that miR-221-3p expression was down-regulated by ox-LDL, and was upregulated by NGR1. We verified that miR-221-3p had anti-apoptosis, anti-inflammation, and anti-oxidative stress effects on ox-LDL-treated
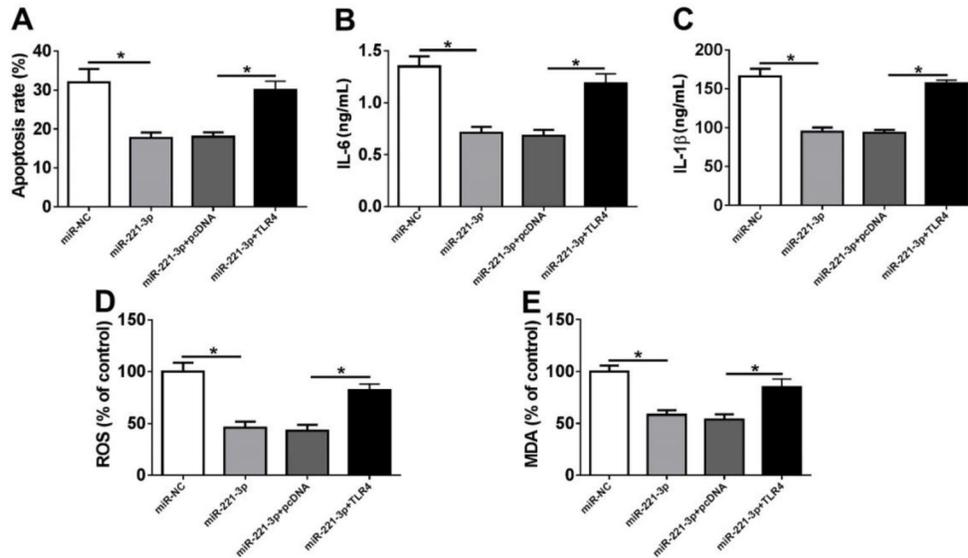
Figure 4. TLR4 overexpression reversed the suppression effects of miR-221-3p on the oxidized low-density lipoprotein (ox-LDL)-induced cellular response in induced human umbilical vein endothelial cells (HUVECs). HUVECs were transfected with miR-221-3p mimic, miR-NC (negative control), miR-221-3p mimic + TLR4, or miR-221-3p mimic + pcDNA, followed by treatment with 50 mg/L ox-LDL. **A**, The apoptosis rates of HUVECs was examined by flow cytometry analysis. **B** and **C**, ELISA was used to determine the levels of interleukin (IL)-6 and IL-1β. **D** and **E**, The levels of reactive oxygen species (ROS) and malondialdehyde (MDA) were determined by assay kits. Data are reported as means ± SD from three independent experiments. *P < 0.05 (ANOVA).
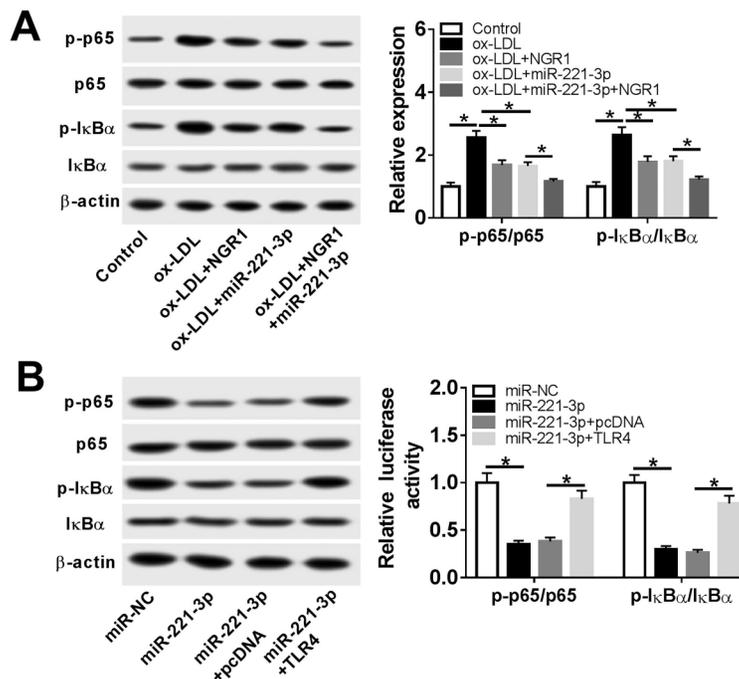

Figure 5. NGR1 inactivated the TLR4/NF-κB pathway in oxidized low-density lipoprotein (ox-LDL)-treated induced human umbilical vein endothelial cells (HUVECs) through miR-221-3p. **A**, HUVECs were transfected with miR-221-3p mimic or miR-NC (negative control), followed by treatment with 50 mg/L ox-LDL or 30 μmol/L NGR1. Western blot analysis was performed to detect the protein levels of p-p65, p65, p-IκBα, and IκBα in HUVECs. **B**, HUVECs were transfected with miR-221-3p mimic, miR-NC, miR-221-3p mimic + pcDNA, or miR-221-3p mimic + TLR4 followed by treatment with 50 mg/L ox-LDL. The protein levels of p-p65, p65, p-IκBα, and IκBα were analyzed by western blot analysis. Data are reported as means ± SD from three independent experiments. *P < 0.05 (ANOVA).

HUVECs. Our findings agreed with those reported by Zhuang et al. (28) that miR-221-3p inhibited ox-LDL-induced oxidative stress in macrophages.

The NF-κB signaling pathway is an essential regulatory downstream factor of TLR4 (29). Baker et al. (30) reported that TLR4 could activate NF-κB and then induce the expression of inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, to participate in the formation and development of AS. Also, the study of Tang et al. (31) showed that the TLR4/NF-κB pathway involved in chronic unpredictable mild stress (CUMS) induced AS in apoE$^{-/-}$ mice by upregulating inflammatory cytokines, indicating that the TLF4/NF-κB pathway participated in regulating the inflammatory response in AS. In this study, we predicted that TLR4 was the target of miR-221-3p, and its expression was negatively correlated with miR-221-3p. Through the detection of the TLR4/NF-κB pathway-related proteins, we found that ox-LDL stimulated the activity of the TLR4/NF-κB pathway, while NGR1 and miR-221-3p overexpression

blocked its activation. Combined with the experimental results, we concluded that NGR1 inhibited the activation of the TLR4/NF-κB pathway by raising miR-221-3p expression.

In conclusion, we found that NGR1 upregulated the expression of miR-221-3p to inhibit apoptosis, inflammation, and oxidative stress in ox-LDL-treated HUVECs by targeting the TLR4/NF-κB pathway. This study showed that NGR1 might be a good strategy for the prevention and treatment of AS and pointed out a novel target for the study of the pathogenesis of AS.

Supplementary Material

Click here to view [pdf].

Acknowledgments

Reference

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