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Original Paper

Notoginsenoside R1 Protects HUVEC Against Oxidized Low Density Lipoprotein (Ox-LDL)-Induced Atherogenic Response via Down-Regulating miR-132

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Key Words
Atherosclerosis • Ox-LDL • Notoginsenoside R1 • HUVEC • miR-132

Abstract
Background/Aims: Radix notoginseng is a well-known traditional Chinese herbal medicine, has extensively pharmacological activities in cardiovascular system. Notoginsenoside R1 (NGR1) is one main active ingredient of Radix notoginseng. The purpose of this study was to evaluate the functional effects of NGR1 on atherosclerosis (AS). ***Methods:*** Human umbilical vascular endothelial cells (HUVECs) were subjected to oxidized low density lipoprotein (ox-LDL), before which cells were preconditioned with NGR1. Cell Counting Kit-8 (CCK-8) assay, flow cytometry, Transwell assay, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot were carried out to assess the impacts of ox-LDL and NGR1 on HUVECs. Besides, the expression of microRNA-132 (miR-132), and the regulatory role of miR-132 in Matrix Gla Protein (MGP) expression were measured by qRT-PCR and Western blot. ***Results:*** NGR1 pre-conditioning prevented ox-LDL-induced apoptosis, migration and overproduction of Monocyte Chemoattractant Protein 1 (MCP-1) and Intercellular Adhesion Molecule 1 (ICAM-1). miR-132 was up-regulated in response to ox-LDL while was down-regulated by NGR1 pre-conditioning. The protective actions of NGR1 in ox-LDL-treated HUVECs were enhanced by miR-132 inhibitor, while were attenuated by miR-132 mimic. Besides, the up-regulated miR-132 could further decrease the expression of MGP, which acted as an anti-migratory and anti-adhesive factor. Furthermore, ox-LDL-induced the activation of c-Jun N-terminal Kinase (JNK) and Nuclear Factor Kappa B (NF-κB) pathways were partially attenuated by NGR1, and were fully eliminated by NGR1 treatment together with MGP overexpression. ***Conclusion:*** NGR1 prevents ox-LDL-induced apoptosis, migration and adhesion-related molecule release in HUVECs possibly via down-regulating miR-132, and subsequent up-regulating MGP.

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Introduction

Atherosclerosis (AS) is the primary pathological base of coronary heart disease, cerebrovascular disease, thromboembolic disease and peripheral artery disease. It is a common disease which is severely harmful to human health, particularly to the elders' health globally [1]. The pathogenesis of AS is complicated and remains unclear. Two theories have been widely-accepted as the initial events involved in the formation of AS, *i.e.*, "response-to-injury" and "chronic inflammation" [2, 3]. Recently, a growing number of studies have focused on investigating the role of oxidized low density lipoprotein (ox-LDL) in AS, in order to expand the understanding of AS pathogenesis. Formation of ox-LDL is an early and crucial step in the development of AS [4], and ox-LDL has been considered as a key biomarker for AS diagnosis [5]. ox-LDL takes part in AS through various mechanisms, for example, its cytotoxic effects on human umbilical vascular endothelial cells (HUVECs) [6]. Based on these reasons, several literatures have applied ox-LDL as a stimuli for establishing an *in vitro* model of AS [7, 8].

Radix notoginseng is a traditional Chinese herbal medicine prepared from roots of *Panax notoginseng* [9]. It has been used for thousands of years in China for hemostasis [9]. Nowadays, a growing number of literatures have focused on the extensively pharmacological activities of Radix notoginseng on the cardiovascular system [10-12]. Panax notoginseng saponins (PNS) are the main active ingredients of Radix notoginseng. With the development of pharmacology, a variety of monomer saponins have been identified, including ginsenoside Rb1, ginsenoside Rg1 and notoginsenoside R1 (NGR1). In regard of AS, a previous study has reported NGR1 as a promising anti-AS agent by modulation of inflammation, oxidative stress, lipid metabolism and the expression of miRNAs [13]. However, the detailed function of NGR1 on AS, and the possibly underlying mechanisms still need to be revealed.

To this end, ox-LDL was used in this study to stimulate HUVECs, before which HUVECs were preconditioned with NGR1. Viability, apoptosis, migration and the expression of adhesion-related molecules were assessed to see the effects of NGR1 on ox-LDL-treated HUVECs. In addition, we investigated the regulatory role of NGR1 in the expression of miR-132, in order to decode the underlying mechanisms of the action. Furthermore, Matrix Gla Protein (MGP), an inhibitor of vessel [14], was recognized as a downstream effector of miR-132 in the present work. The reason for why we choose miR-132, but not other miRNAs is that: miR-132 has been reported as a highly expressed miRNA in atherosclerotic lesions in ApoE-/- mice [15]. Besides, NGR1 could slightly decrease miR-132 expression in ApoE-/- mice [13]. The findings of this study will further suggest NGR1 as a promising agent for the prevention and treatment of AS.

Materials and Methods

Cell treatment

HUVECs were purchased from ATCC (Manassas, VA). The cells were routinely cultured in RPMI-1640 medium (Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS, Gibco), 30 μg/ml endothelial cell growth supplement (Collaborative Research, Lexington, MA), and 0.1 mg/ml heparin (Gibco) at 37°C in 5% CO_2. HUVECs under passage 7 were used throughout the study.

ox-LDL was prepared by AngYuBio (Shanghai, China). For the establishment of *in vitro* model of AS, HUVECs were treated by 100 mg/l of ox-LDL for 24 h.

NGR1 (HPLC⩾98%) purchased from Linyi azeroth biotechnology CO. LTD. (Linyi, China) was dissolved in DMSO (Sigma, St. Louis, MO), and was diluted with the culture medium so that the final concentration of DMSO was less than 0.1%. HUVECs were treated by various doses of NGR1 for 24 h before ox-LDL treatment. The cells received treatment of 0.1% DMSO were considered as a blank control.

Assessment of cell viability

HUVECs in 96-well plates (5×10^3 cells per well) were treated by NGR1 and/or ox-LDL, after which the culture medium was removed. The cells were washed twice with phosphate buffer saline (PBS, Beyotime, Shanghai, China), and then 10 μl CCK-8 solution (Dojindo Molecular Technologies, Kyushu, Japan) was added into each well. The plates were incubated at 37°C for 2 h, then the absorbance of each well was detected by an ELISA reader (Bio-Rad, Hercules, CA) at 450 nM.

Assessment of apoptosis

After the treatment of NGR1 and ox-LDL, HUVECs in 6-well plates (5×10^5 cells per well) were collected by 0.25% trypsin (Gibico). Apoptosis was measured by the Annexin V-FITC Apoptosis Detection Kit (Beyotime). At least 1×10^5 cells of each sample were resuspended in 100 μl Binding Buffer containing 10 μl Annexin V-FITC. After 30 min of incubation over ice in the dark, 5 μl PI and 400 μl ice-cold PBS was added. The samples were then analyzed by a flow cytometer (Beckman Coulter, San Jose, CA). Annexin-V positive and PI-negative cells were considered as apoptotic cells.

Migration assay

HUVECs (5×10^4) in 200 μl non-serum medium were placed in the upper chamber of the 24-well Transwell plate (8-μm pore insert, Costar-Corning, New York). The lower side of the plates was filled with 600 μl complete medium. After treating with NGR1 and/or ox-LDL, the cells were stained with crystal violet and were counted microscopically. In order to rule out the impacts of proliferation on the calculation of migratory rate, relative migration was calculated as following formula: cell number in lower chamber/(cell number in lower and upper chambers) × 100%. Five fields of vision were randomly selected for the calculation of the relative migration.

Cell transfection

The mimic, inhibitor and scrambled control (NC) specific for human miR-132 were purchased from GenePharma (Shanghai, China). Full-length wide-type of MGP was inserted into the pc-DNA3.1 plasmid (Invitrogen, Carlsbad, CA) to form a MGP expression vector. The empty pc-DNA3.1 plasmid was used as a blank control. For MGP silencing, shRNA specific for MGP synthetized by RiboBio (Guangzhou, China) was constructed into pGPU6/Neo plasmid (GenePharma). pGPU6/Neo plasmid with non-targeting sequences was transfected as a blank control. All transfections were conducted in 6-well plates with non-serum medium by using Lipofectamine 3000 (Invitrogen). At 48 h of transfection, cells were collected.

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

Total RNA in HUVECs was extracted by using Trizol reagent (Life Technologies Corporation, Carlsbad, CA). For detection of miRNA expression, reverse transcription was performed by PrimeScript Reverse Transcriptase (Takara, Dalian, China). qRT-PCR was conducted by Taqman Universal Master Mix II (Applied Biosystems, Foster City, CA). For detection of the expression of Monocyte Chemoattractant Protein 1 (MCP-1), Intercellular Adhesion Molecule 1 (ICAM-1), and MGP, cDNA was synthetized by Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). qRT-PCR was performed by FastStart Universal SYBR Green Master (Roche). The internal control for miRNAs was U6, and β-actin for MCP-1, ICAM-1, and MGP.

Western blot

Total protein in HUVECs was extracted by using RIPA Lysis Buffer (Beyotime), and the concentration of the whole-cell extract was detected by the BCA™ Protein Assay Kit (Pierce, Appleton, WI). Protein in the whole-cell extract was separated by the SDS-PAGE, and was transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were blocked in QuickBlock™ Blocking Buffer (Beyotime) for 30 min at room temperature, and then probed at 4°C overnight with primary antibodies: MCP-1 (Cat # M2MCP1BI, Thermo Fisher Scientific, Waltham, MA), ICAM-1 (Cat # MA5407, Thermo Fisher Scientific), p53 (Cat # ab26, Abcam, Cambridge, MA), cleaved caspase-3 (Cat # ab2302, Abcam), pro caspase-3 (Cat # ab44976, Abcam), MGP (Cat # ab192396, Abcam), c-Jun N-terminal Kinase (Jnk) (Cat # ab199380, Abcam), p-Jnk (Cat # ab47337, Abcam), c-Jun (Cat # ab32137, Abcam), p-c-Jun (Cat # ab32385, Abcam), p65 (Cat # ab32536, Abcam), p-p65 (Cat # ab86299, Abcam), IκBα (Cat # ab7217, Abcam), p-IκBα (Cat # ab133462, Abcam), and β-actin (Cat #

ab8226, Abcam). Following incubation with the secondary antibodies for 1 h at room temperature, protein bands were visualized by enhanced chemiluminescence method. Intensity of the bands was analyzed by Image Lab™ Software (Bio-Rad, Hercules, CA).

Statistics

Data are presented as mean ± SD from three independent experiments. Statistical differences between two groups were tested by Student *t* test, and statistical differences between three or more were determined by a one-way analysis of variance (ANOVA). SPSS 19.0 software (SPSS Inc., Chicago, IL) was used in this procedure. p-values of < 0.05 were considered as significant results.

Results

ox-LDL promotes apoptosis, migration and adhesion-related molecule release of HUVECs

HUVECs were subjected to 100 mg/l ox-LDL for 24 h. As a result, cell viability was significantly reduced by ox-LDL to 53.87% ($p<0.05$, Fig. 1A). Besides, apoptosis was induced by ox-LDL. As shown in Fig. 1B-1C, apoptotic cell rate was increased from 2.92% to 21.02% ($p<0.05$), and the protein expression of p53 was up-regulated and caspase-3 was cleaved remarkably. Fig. 1D showed that the relative migration was significantly increased by ox-LDL treatment ($p<0.05$). Further, qRT-PCR and Western blot analysis were performed to assess the expression changes of adhesion-related molecules. Results in Fig. 1E-1G showed that, the mRNA and protein levels of MCP-1 and ICAM-1 were both highly expressed in response to ox-LDL ($p<0.05$).

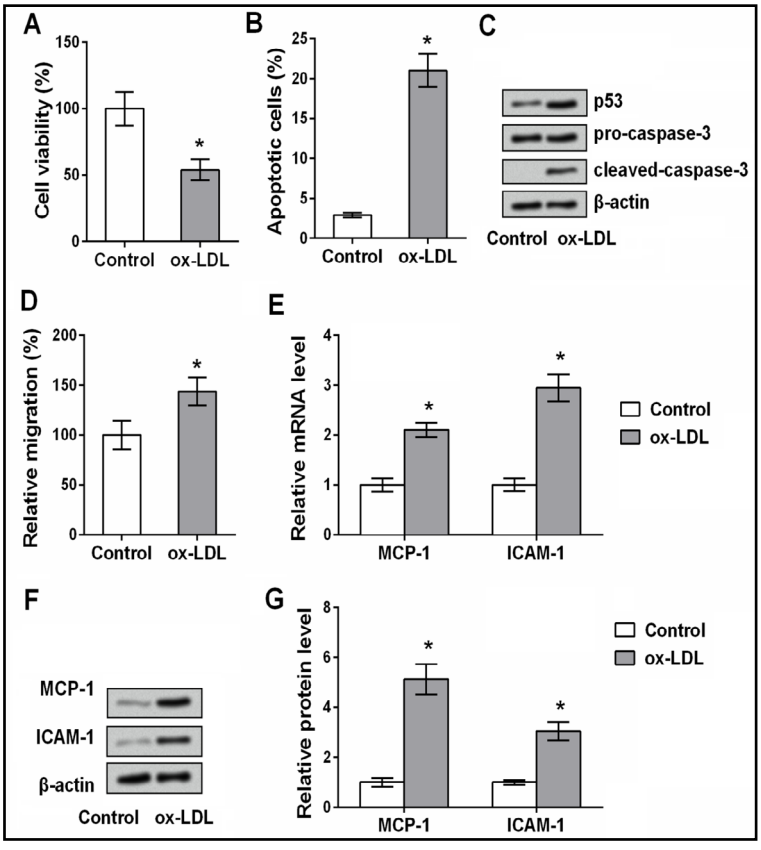

Fig. 1. Effects of oxidized low density lipoprotein (ox-LDL) on HUVECs. (A) Viability, (B) the rate of apoptotic cells, (C) expression of apoptosis-related proteins, (D) relative migration, (E) mRNA levels of MCP-1 and ICAM-1, and (F-G) protein levels of MCP-1 and ICAM-1, in HUVECs after 24 h of exposure to 100 mg/l ox-LDL. * $p<0.05$ compared to the control group.

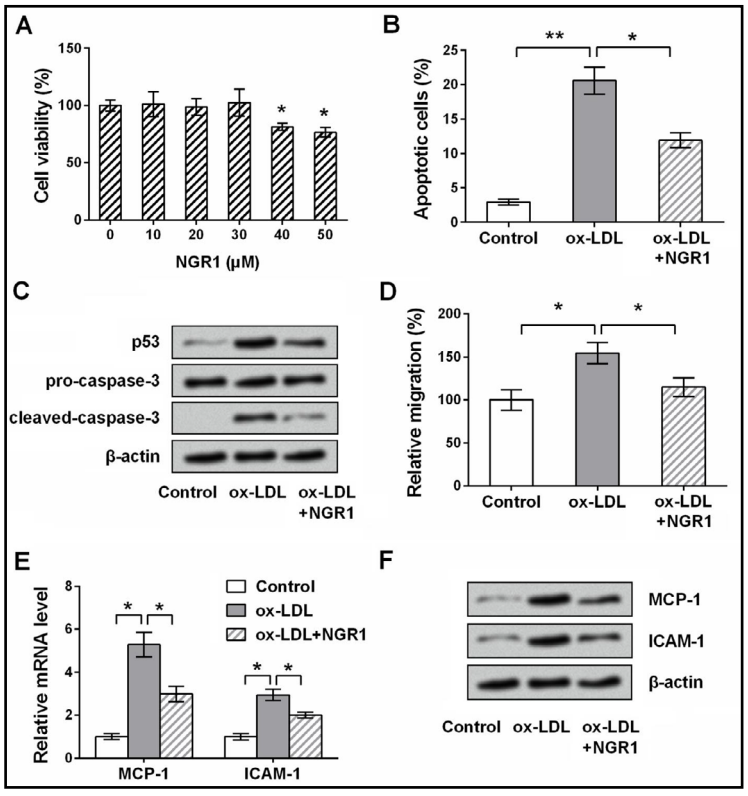

Fig. 2. Effects of notoginsenoside R1 (NGR1) on oxidized low density lipoprotein (ox-LDL)-injured HUVECs. (A) Viability of HUVECs treated by various doses of NGR1 for 24 h. * p<0.05 compared to the group without NGR1 treatment. (B) The rate of apoptotic cells, (C) expression of apoptosis-related proteins, (D) relative migration, (E) mRNA and (F) protein levels of MCP-1 and ICAM-1, in HUVECs after pre-conditioning with 30 μM NGR1 for 24 h, and subsequent exposure of 100 mg/l ox-LDL for 24 h. * p<0.05 compared to the indicated group.

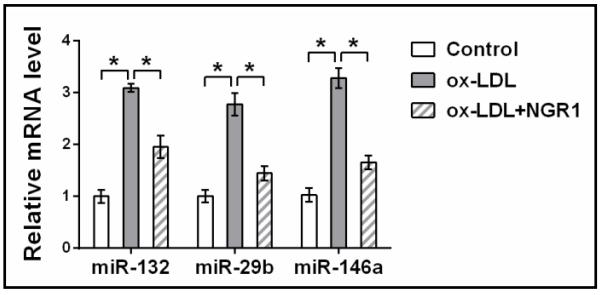

Fig. 3. Effects of notoginsenoside R1 (NGR1) on miRNA expression. RNA levels of miR-132, miR-29b and miR-146a in HUVECs after pre-conditioning with 30 μM NGR1 for 24 h, and subsequent exposure of 100 mg/l ox-LDL for 24 h. * p<0.05 compared to the indicated group.

NGR1 prevents ox-LDL-induced apoptosis, migration and adhesion-related molecule release

HUVECs were pre-conditioned with various doses of NGR1 for 24 h, after which the inhibition of viability was observed at 40 and 50 μM doses of NGR1 (p<0.05, Fig. 2A). 30 μM was selected as a NGR1-treating condition for use in the following experiments. HUVECs were in turn treated by NGR1 and ox-LDL, and we found that the apoptosis induced by ox-LDL was attenuated by NGR1 pre-conditioning (p<0.05, Fig. 2B-2C). In addition, the impacts of ox-LDL on HUVECs migration and MCP-1 and ICAM-1 expression were attenuated when HUVECs were pre-conditioned with NGR1 (ox-LDL group vs. ox-LDL + NGR1 group, p<0.05, Fig. 2D-2F).

NGR1 inhibits the expression of miR-132, miR-29b and miR-146a

miR-132, miR-29b and miR-146a are three pro-inflammatory miRNAs, which can be up-regulated by ox-LDL or ApoE knockout [15-17]. The present work studied the expression changes of these miRNAs, in order to reveal whether NGR1 functioned to HUVECs via a miRNA-dependent pathway. By performing qRT-PCR, we found that miR-132, miR-29b and

miR-146a were all significantly up-regulated in ox-LDL-treated cell ($p<0.05$), and the up-regulations induced by ox-LDL were partially eliminated by NGR1 preconditioning ($p<0.05$, Fig. 3). miR-132 was selected for the follow-up experiments.

NGR1 prevents ox-LDL-induced atherogenic response through down-regulation of miR-132

The specific mimic and inhibitor for miR-132 was transfected into HUVECs respectively, to see the involvement of miR-132 in the pharmacological activities of NGR1. As shown in Fig. 4A, compared to the NC group, the expression of miR-132 was significantly overexpressed by mimic transfection, while was significantly suppressed by inhibitor transfection (both $p<0.05$). The inhibition of apoptosis induced by NGR1 in ox-LDL-treated HUVECs was enhanced by miR-132 inhibitor, while was attenuated by miR-132 mimic ($p<0.05$, Fig. 4B-4C). Also, NGR1-induced migration inhibition, and MCP-1 and ICAM-1 down-regulations were enhanced by miR-132 inhibitor, and were attenuated by miR-132 mimic ($p<0.05$, Fig. 4D-4F).

MGP is down-regulated by miR-132 mimic

MGP, an inhibitor of vessel [14], has been regarded as a biomarker [18] and potential treatment target for AS [19]. Thus, the expression changes of MGP in response to miR-132 dysregulation were detected, to evaluate the involvement of miR-132 expression in the risk of AS. Surprisingly, we found that both the mRNA and protein levels of MGP were up-regulated

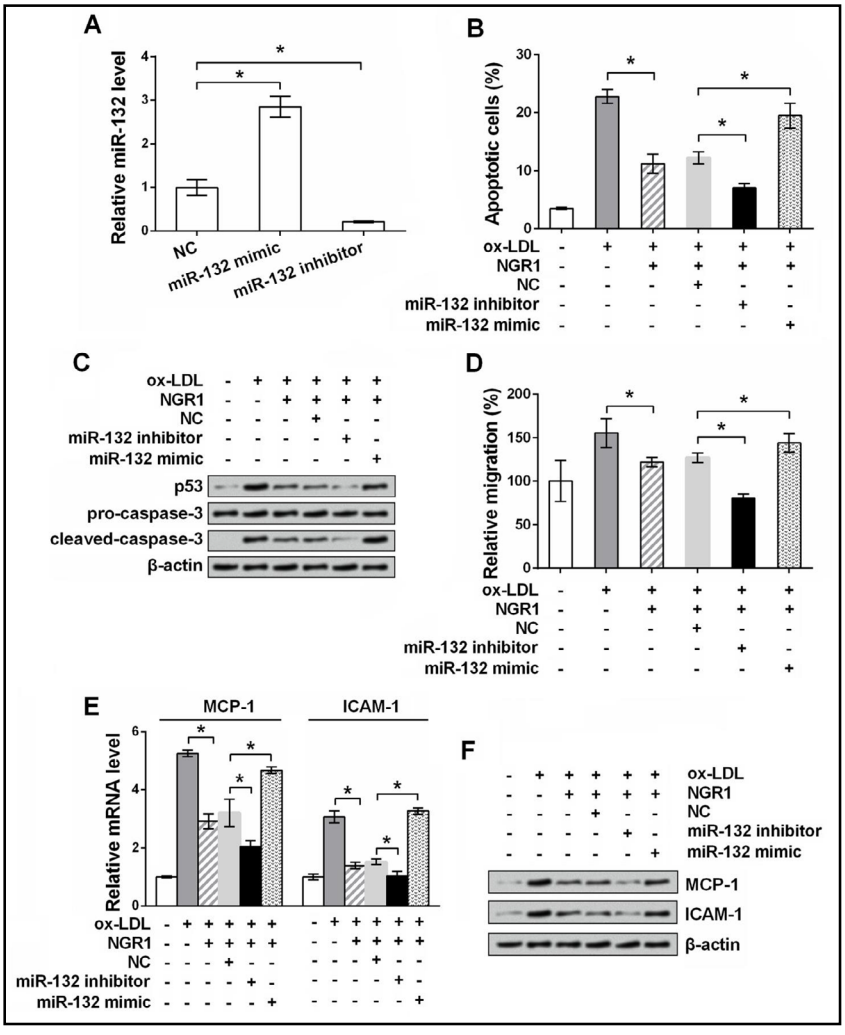

Fig. 4. Effects of miR-132 on notoginsenoside R1 (NGR1)- and oxidized low density lipoprotein (ox-LDL)-treated HUVECs. (A) RNA levels of miR-132 in HUVECs after transfection with mimic, inhibitor and scrambled control (NC) for miR-132. (B) The rate of apoptotic cells, (C) expression of apoptosis-related proteins, (D) relative migration, (E) mRNA and (F) protein levels of MCP-1 and ICAM-1. HUVECs were transfected with miR-132 mimic, miR-132 inhibitor or NC, and then were in turn treated by NGR1 and ox-LDL. * $p<0.05$ compared to the indicated group.

by miR-132 inhibitor transfection, while were down-regulated by mimic transfection ($p<0.05$, Fig. 5A-5B). Next, *in silico* analysis was carried out to predict whether MGP was a target gene for miR-132. By searching on Targetscan and microRNA. org online databases, no targeting relationship between miR-132 and MGP was found. Thus, we inferred that the relationship between miR-132 and MGP is very complicated that some other factors may be involved.

MGP prevents ox-LDL-induced migration and adhesion-related molecule release

The expression of MGP in HUVECs was altered by vector or shRNA transfection. As shown in Fig. 6A-6B, pc-MGP transfection significantly elevated MGP expression, and sh-MGP transfection reduced MGP expression ($p<0.05$). Then, we found that ox-LDL-induced increases in migration and the expression of MCP-1 and ICAM-1 were attenuated by pc-MGP and were enhanced by sh-MCP ($p<0.05$, Fig. 6C-6E).

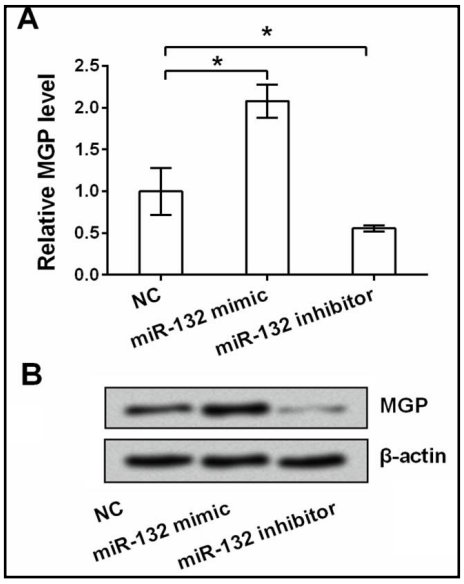

Fig. 5. Effects of miR-132 dysregulation on MGP expression. (A) mRNA and (B) protein levels of MGP in HUVECs after transfection with miR-132 mimic, miR-132 inhibitor or scrambled control (NC). * $p<0.05$ compared to the indicated group.

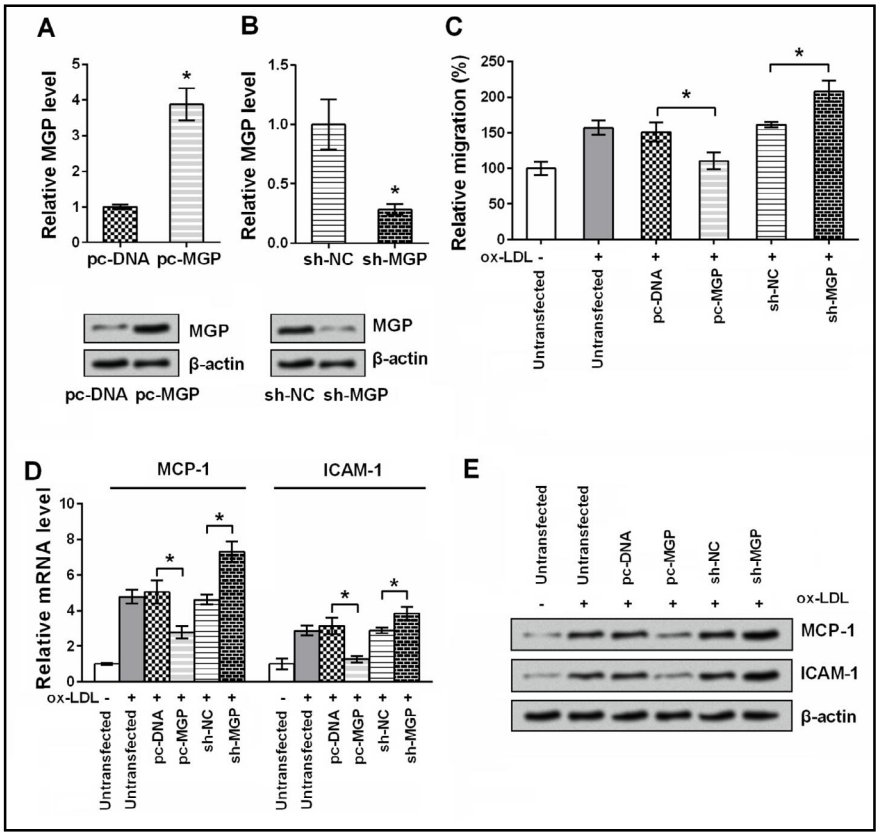

Fig. 6. Effects of MGP dysregulation on oxidized low density lipoprotein (ox-LDL)-treated HUVECs. (A-B) mRNA and protein levels of MGP in HUVECs after transfection with pc-DNA, pc-MGP, sh-NC or sh-MGP. (C) Relative migration, (D) mRNA and (E) protein levels of MCP-1 and ICAM-1 in HUVECs after transfection with pc-DNA, pc-MGP, sh-NC or sh-MGP, and subsequent exposure of ox-LDL. * $p<0.05$ compared to the indicated group.

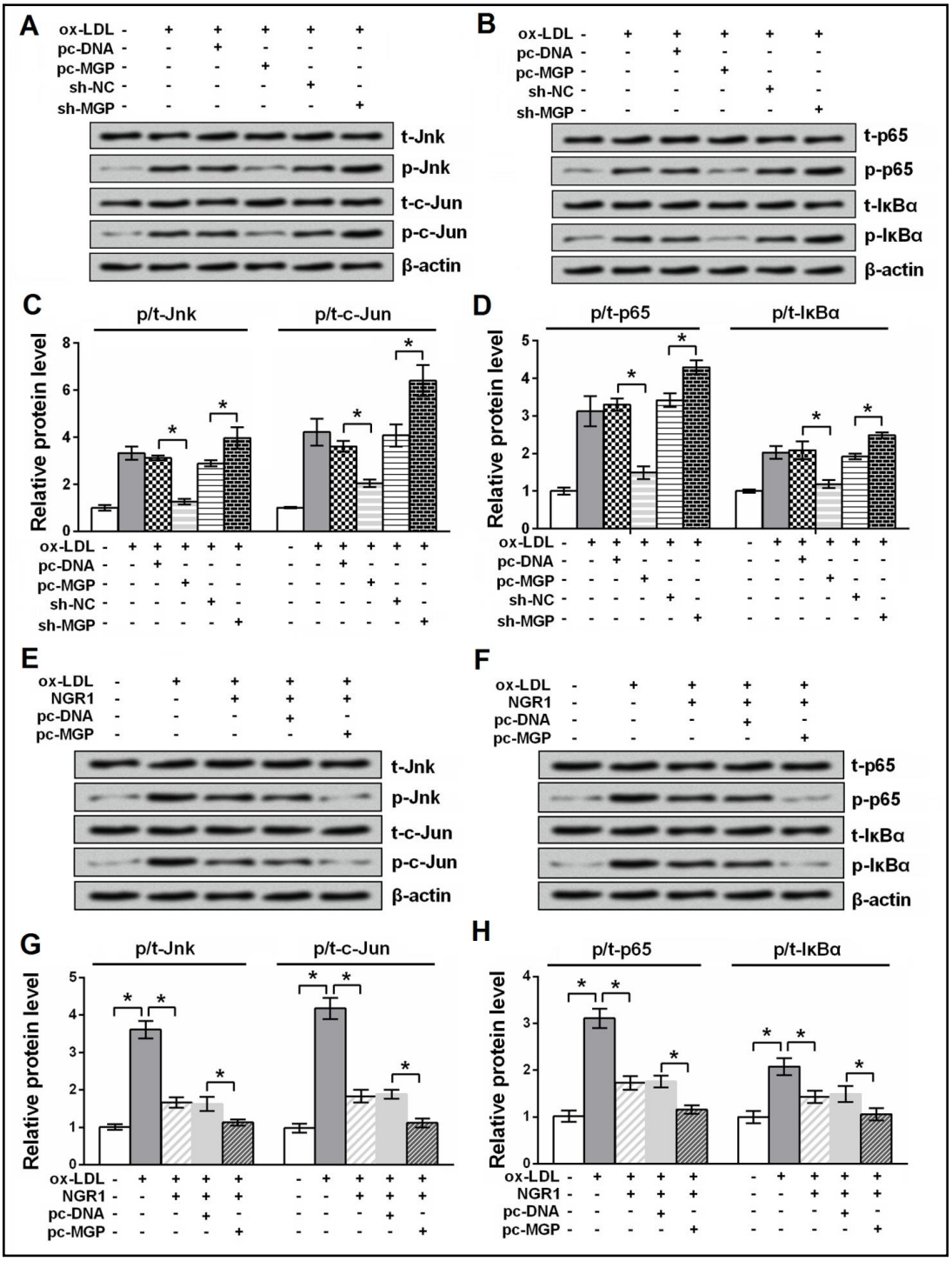

Fig. 7. Effects of notoginsenoside R1 (NGR1) treatment and MGP dysregulation on JNK and NF-κB pathways. (A, C) Protein levels of Jnk, c-Jun, and (B, D) protein levels of p65, IκBα in HUVECs after transfection with pc-DNA, pc-MGP, sh-NC or sh-MGP, and subsequent exposure of ox-LDL. (E, G) Protein levels of Jnk, c-Jun, and (F, H) protein levels of p65, IκBα in HUVECs after transfection with pc-DNA/pc-MGP, and subsequently treated by NGR1 and ox-LDL. * $p<0.05$ compared to the indicated group.

MGP deactivates JNK and NF-κB pathways

Furthermore, we concerned the regulatory role of MGP in JNK and NF-κB pathways, to reveal the underlying mechanism of which MGP protected HUVECs. We found that ox-LDL-induced the activation of JNK and NF-κB pathways, as Jnk, c-Jun, p65 and IκBα were all significantly phosphorylated by ox-LDL treatment ($p<0.05$, Fig. 7A-7D). More importantly, the activation of these two signaling was blocked by MGP overexpression while was accelerated by MGP suppression ($p<0.05$). Moreover, the effects of NGR1 treatment together with MGP overexpression on the activation of JNK and NF-κB pathways induced by ox-LDL were tested. As shown in Fig. 7E-7H, the activation of these two signaling induced by ox-LDL was attenuated by NGR1 ($p<0.05$), however NGR1 together with MGP overexpression could fully eliminate the activation ($p<0.05$).

Discussion

The formation of plaques is a multi-stage and chronic process, in which a series of cellular events occurring, including vascular endothelial cell damage, adhesion of monocytes and lymphocytes, invasion into endothelium, foam cell transformation, fatty streak formation, and smooth muscle cell migration and hyperplasia. This study mainly focused on the effects of NGR1 on HUVECs damage induced by ox-LDL, to preliminary evaluate the potential usage of NGR1 for AS. We found that NGR1 pre-conditioning prevented ox-LDL-induced apoptosis, migration and the overproduction of MCP-1 and ICAM-1. We additionally found that miR-132 was up-regulated in response to ox-LDL while was down-regulated by NGR1 pre-conditioning. The up-regulated miR-132 further decreased the expression of MGP, which in turn repressed the activation of JNK and NF-κB pathways.

Vascular endothelial cell damage is one of the initial events of the development of AS. High concentration of ox-LDL induces cell apoptosis via multiple pathways, including ROS generation, caspase, and alterations of apoptosis-related gene expressions [20]. This was also confirmed in this study, that 100 mg/l of ox-LDL significantly increased the rate of apoptotic cells, and remarkably cleaved caspase-3. Besides, ox-LDL up-regulated the expression of p53, which is a controller of apoptosis in malignant cells [21] and AS-related cell types [22]. Although the cytotoxic effects of ox-LDL on vascular endothelial cells have been well-established, the exact role of ox-LDL in migratory capacity is still confusing. Sun *et al.*, demonstrated that ox-LDL inhibited the migration of HUVECs by performing Transwell assay, without excluding the proliferation-inhibitory impacts of ox-LDL on the number of the migrated cells [23]. However, the opposite effect of ox-LDL has been reported by Bao *et al.*, in the same cell line [24]. Consistent with the findings of Bao *et al.*, we suggested that ox-LDL promoted HUVECs migration by ruling out proliferation-inhibitory impact. Moreover, we additionally found that ox-LDL increased the release of two adhesion-related molecules (MCP-1 and ICAM-1), further confirmed the migration-promoting role of ox-LDL in HUVECs. MCP-1, a chemotactic protein for monocytes [25], contributes in monocyte-endothelium adhesion and subsequent transendothelial migration [26].

The anti-AS effect of PNS has been sporadically studied. A previous study performed in ApoE-/- mice demonstrated that, PNS decreased the serum ox-LDL level and the ratio of plaque area to vessel area [27]. Another investigation showed that PNS protected HUVECs against ox-LDL-induced injury partially via down-regulation of ICAM-1 [28]. However, to our best of knowledge, only one literature has reported the anti-AS effect of NGR1 on an ApoE-/- mice model [13]. Herein, the same conclusion was drawn from the *in vitro* model made by ox-LDL in HUVECs. Also, the protective effects of NGR1 against ox-LDL-induced inflammation have been mentioned in vascular endothelial cells [29]. All these suggested the potential usage of NGR1 in AS.

It is well-known that AS is an inflammatory disease [3], with expression changes of multiple of miRNAs [30]. Among which, miR-132, miR-29b and miR-146a have been emerged as key regulators in inflammation-mediated processes [31-33]. Besides, previous studies have demonstrated that miR-29b and miR-146a could be up-regulated by ox-LDL, and miR-132 could be up-regulated by ApoE knockout [15-17]. The present work showed that all of these three miRNAs were up-regulated in ox-LDL-treated HUVECs, and were down-regulated by NGR1 preconditioning. It seems that the effects of NGR1 on miR-132 expression are nonspecific. The impacts of NGR1 on miRNA expression might be via both genomic (transcriptional) and non-genomic mechanisms of action. Furthermore, we selected miR-132 for use in the follow-up rescue assays, since miR-132 has been reported to promote TNF-α-induced inflammation and cell death of HUVECs [34]. Besides, Kumarswamy and his colleagues have mentioned that deletion of miR-212/132 cluster increased endothelial vasodilatory function [35]. We found that the protective effects of NGR1 against ox-LDL-induced injury in HUVECs were attenuated by miR-132 mimic. These data implied that NGR1 functioned to HUVECs possibly via down-regulation of miR-132. However, further studies are required to verify whether miR-29b and miR-146a are two other downstream effectors of NGR1.

MGP is an important inhibitor of vessel and cartilage calcification that is highly expressed in human atherosclerotic plaques [14]. Elevated level of MGP in serum is associated with the increased risk of cardiovascular morbidity and mortality [18]. In this work, MGP was found to be up-regulated by miR-132 inhibitor, indicating MGP might be one of the downstream genes of miR-132. Besides, by searching on Targetscan and microRNA.org online databases, no targeting relationship between miR-132 and MGP was found. Thus, we inferred that the relationship between miR-132 and MGP is very complicated that some other factors may be involved. We additionally found that MGP overexpression could help HUVECs to maintain non-migration and non-adhesion in ox-LDL-treated condition. Similar findings were reported by Yao *et al.*, that MGP overexpression reduced atherosclerotic lesion size and inflammation in ApoE-/- mice [36].

A number of signaling pathways are highly relevant to AS [37], among which JNK and NF-κB have been widely-accepted as key pathways responsible for AS [38, 39]. Specific inhibition of the activity of JNK and NF-κB pathways may be effectively therapeutic approach to prevent and/or treat AS [38, 39]. In this paper, we showed that ox-LDL-induced the activation of JNK and NF-κB pathways, and NGR1 attenuated the activation of these two signaling. This phenomenon was in line with previous studies [29, 40], indicating NGR1 exerted anti-AS effects through inhibiting ox-LDL-induced JNK and NF-κB activation. More interestingly, NGR1 together with MGP overexpression could fully eliminate the activation of these two signaling. Thus, we inferred that NGR1 protected HUVECs against ox-LDL possibly via regulating MGP, which subsequently inhibiting JNK and NF-κB pathways.

Conclusion

In conclusion, we show here that NGR1 prevents ox-LDL-induced apoptosis, migration and adhesion-related molecule release in HUVECs. The protective actions of NGR1 may be via down-regulation of miR-132, and subsequent up-regulation of MGP. The findings provide *in vitro* evidence that NGR1 exerted anti-AS effect.

Disclosure Statement

The authors declare that they have no competing interests.

References

1 Collins AR, Lyon CJ, Xia X, Liu JZ, Tangirala RK, Yin F, Boyadjian R, Bikineyeva A, Pratico D, Harrison DG, Hsueh WA: Age-accelerated atherosclerosis correlates with failure to upregulate antioxidant genes. Circ Res 2009;104:e42-54.
2 Ross R: The pathogenesis of atherosclerosis--an update. N Engl J Med 1986;314:488-500.
3 Libby P, Ridker PM, Maseri A: Inflammation and atherosclerosis. Circulation 2002;105:1135-1143.
4 Bergmark C, Wu R, de Faire U, Lefvert AK, Swedenborg J: Patients with early-onset peripheral vascular disease have increased levels of autoantibodies against oxidized LDL. Arterioscler Thromb Vasc Biol 1995;15:441-445.
5 Maggi E, Finardi G, Poli M, Bollati P, Filipponi M, Stefano PL, Paolini G, Grossi A, Clot P, Albano E: Specificity of autoantibodies against oxidized LDL as an additional marker for atherosclerotic risk. Coron Artery Dis 1993;4:1119-1122.
6 Claise C, Chalas J, Edeas M, Abella A, Khalfoun Y, Laurent D, Lindenbaum A: Comparison of oxidized low-density lipoprotein toxicity on EA.hy 926 cells and human vein endothelial cells: influence of antioxidant systems. Cell Mol Life Sci 1997;53:156-161.
7 Qi JC, Liu PG, Wang C, Zheng AD, Wan Z: Tacrolimus protects vascular endothelial cells from injuries caused by Ox-LDL by regulating endoplasmic reticulum stress. Eur Rev Med Pharmacol Sci 2017;21:3966-3973.
8 Liu J, Yao S, Wang S, Jiao P, Song G, Yu Y, Zhu P, Qin S: D-4F, an apolipoprotein A-I mimetic peptide, protects human umbilical vein endothelial cells from oxidized low-density lipoprotein-induced injury by preventing the downregulation of pigment epithelium-derived factor expression. J Cardiovasc Pharmacol 2014;63:553-561.
9 Yang X, Xiong X, Wang H, Wang J: Protective effects of panax notoginseng saponins on cardiovascular diseases: a comprehensive overview of experimental studies. Evid Based Complement Alternat Med 2014;2014:204840.
10 Li W, Li X, Du Q, Li F, Zhu Y, Liu Y, Ma J, Wan L, Li F, Zhang S: Effect of tongluojiunao injection made from sanqi (Radix Notoginseng) and zhizi (Fructus Gardeniae) on brain microvascular endothelial cells and astrocytes in an *in vitro* ischemic model. J Tradit Chin Med 2014;34:725-732.
11 Shang Q, Xu H, Liu Z, Chen K, Liu J: Oral Panax notoginseng Preparation for Coronary Heart Disease: A Systematic Review of Randomized Controlled Trials. Evid Based Complement Alternat Med 2013;2013:940125.
12 Yuan Z, Liao Y, Tian G, Li H, Jia Y, Zhang H, Tan Z, Li X, Deng W, Liu K, Zhang Y: Panax notoginseng saponins inhibit Zymosan A induced atherosclerosis by suppressing integrin expression, FAK activation and NF-kappaB translocation. J Ethnopharmacol 2011;138:150-155.
13 Jia C, Xiong M, Wang P, Cui J, Du X, Yang Q, Wang W, Chen Y, Zhang T: Notoginsenoside R1 attenuates atherosclerotic lesions in ApoE deficient mouse model. PLoS One 2014;9:e99849.
14 Shanahan CM, Cary NR, Metcalfe JC, Weissberg PL: High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. J Clin Invest 1994;93:2393-2402.
15 Xiong M, Jia C, Cui J, Wang P, Du X, Yang Q, Zhu Y, Wang W, Zhang T, Chen Y: Shexiang Tongxin dropping pill attenuates atherosclerotic lesions in ApoE deficient mouse model. J Ethnopharmacol 2015;159:84-92.
16 Chen KC, Wang YS, Hu CY, Chang WC, Liao YC, Dai CY, Juo SH: OxLDL up-regulates microRNA-29b, leading to epigenetic modifications of MMP-2/MMP-9 genes: a novel mechanism for cardiovascular diseases. Faseb j 2011;25:1718-1728.
17 Li Z, Wang S, Zhao W, Sun Z, Yan H, Zhu J: Oxidized low-density lipoprotein upregulates microRNA-146a via JNK and NF-kappaB signaling. Mol Med Rep 2016;13:1709-1716.
18 Buyukterzi Z, Can U, Alpaydin S, Guzelant A, Karaarslan S, Mustu M, Kocyigit D, Gurses KM: Enhanced serum levels of matrix Gla protein and bone morphogenetic protein in acute coronary syndrome patients. J Clin Lab Anal 2017;23:10.1002/jcla.22278.
19 Mayer O, Jr.: Matrix Gla protein as natural inhibitor of vascular calcification and potential treatment target. Cas Lek Cesk 2016;155:13-16.
20 Salvayre R, Auge N, Benoist H, Negre-Salvayre A: Oxidized low-density lipoprotein-induced apoptosis. Biochim Biophys Acta 2002;1585:213-221.
21 Aubrey BJ, Kelly GL, Janic A, Herold MJ, Strasser A: How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression? Cell Death Differ 2018;25:104-113.

22 Mercer J, Mahmoudi M, Bennett M: DNA damage, p53, apoptosis and vascular disease. Mutat Res 2007;621:75-86.
23 Sun H, Zhu X, Zhou Y, Cai W, Qiu L: C1q/TNF-Related Protein-9 Ameliorates Ox-LDL-Induced Endothelial Dysfunction via PGC-1alpha/AMPK-Mediated Antioxidant Enzyme Induction. Int J Mol Sci 2017;18:1097.
24 Bao MH, Li GY, Huang XS, Tang L, Dong LP, Li JM: Long non-coding RNA LINC00657 acting as miR-590-3p sponge to facilitate low concentration oxidized low-density lipoprotein-induced angiogenesis. Mol Pharmacol 2018;93:368-375.
25 Randolph GJ, Furie MB: A soluble gradient of endogenous monocyte chemoattractant protein-1 promotes the transendothelial migration of monocytes *in vitro*. J Immunol 1995;155:3610-3618.
26 Takahashi M, Masuyama J, Ikeda U, Kitagawa S, Kasahara T, Saito M, Kano S, Shimada K: Suppressive role of endogenous endothelial monocyte chemoattractant protein-1 on monocyte transendothelial migration *in vitro*. Arterioscler Thromb Vasc Biol 1995;15:629-636.
27 Liu G, Wang B, Zhang J, Jiang H, Liu F: Total panax notoginsenosides prevent atherosclerosis in apolipoprotein E-knockout mice: Role of downregulation of CD40 and MMP-9 expression. J Ethnopharmacol 2009;126:350-354.
28 Qin JH, Zhu LQ, Cui W: Effects of panax notoginseng saponins on expression of intercellular adhesion molecule-1 in human umbilical vein endothelial cells injury induced by oxidized low-density lipoprotein. Zhongguo Zhong Xi Yi Jie He Za Zhi 2008;28:1096-1099.
29 Su P, Du S, Li H, Li Z, Xin W, Zhang W: Notoginsenoside R1 inhibits oxidized low-density lipoprotein induced inflammatory cytokines production in human endothelial EA.hy926 cells. Eur J Pharmacol 2016;770:9-15.
30 Laffont B, Rayner KJ: MicroRNAs in the Pathobiology and Therapy of Atherosclerosis. Can J Cardiol 2017;33:313-324.
31 Marques-Rocha JL, Samblas M, Milagro FI, Bressan J, Martinez JA, Marti A: Noncoding RNAs, cytokines, and inflammation-related diseases. Faseb j 2015;29:3595-3611.
32 Yuan H, Ma J, Li T, Han X: MiR-29b aggravates lipopolysaccharide-induced endothelial cells inflammatory damage by regulation of NF-kappaB and JNK signaling pathways. Biomed Pharmacother 2018;99:451-461.
33 Habibi F, Ghadiri Soufi F, Ghiasi R, Khamaneh AM, Alipour MR: Alteration in Inflammation-related miR-146a Expression in NF-KB Signaling Pathway in Diabetic Rat Hippocampus. Adv Pharm Bull 2016;6:99-103.
34 Zhang L, Huang D, Wang Q, Shen D, Wang Y, Chen B, Zhang J, Gai L: MiR-132 inhibits expression of SIRT1 and induces pro-inflammatory processes of vascular endothelial inflammation through blockade of the SREBP-1c metabolic pathway. Cardiovasc Drugs Ther 2014;28:303-311.
35 Kumarswamy R, Volkmann I, Beermann J, Napp LC, Jabs O, Bhayadia R, Melk A, Ucar A, Chowdhury K, Lorenzen JM, Gupta SK, Batkai S, Thum T: Vascular importance of the miR-212/132 cluster. Eur Heart J 2014;35:3224-3231.
36 Yao Y, Bennett BJ, Wang X, Rosenfeld ME, Giachelli C, Lusis AJ, Bostrom KI: Inhibition of bone morphogenetic proteins protects against atherosclerosis and vascular calcification. Circ Res 2010;107:485-494.
37 Hopkins PN: Molecular biology of atherosclerosis. Physiol Rev 2013;93:1317-1542.
38 Bryk D, Olejarz W, Zapolska-Downar D: Mitogen-activated protein kinases in atherosclerosis. Postepy Hig Med Dosw (Online) 2014;68:10-22.
39 Pateras I, Giaginis C, Tsigris C, Patsouris E, Theocharis S: NF-kappaB signaling at the crossroads of inflammation and atherogenesis: searching for new therapeutic links. Expert Opin Ther Targets 2014;18:1089-1101.
40 Tu L, Wang Y, Chen D, Xiang P, Shen J, Li Y, Wang S: Protective Effects of Notoginsenoside R1 via Regulation of the PI3K-Akt-mTOR/JNK Pathway in Neonatal Cerebral Hypoxic-Ischemic Brain Injury. Neurochem Res 2018;43:1210-1226.