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Probiotic administration of Lactobacillus rhamnosus GR-1 attenuates atherosclerotic plaque formation in ApoE$^{-/-}$ mice fed with a high-fat diet

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Abstract. – OBJECTIVE: To investigate the effect of *Lactobacillus rhamnosus* GR-1 on atherosclerotic progression in apolipoprotein-E knockout (ApoE$^{-/-}$) mice fed with a high-fat diet and the underlying mechanisms of its action.

MATERIALS AND METHODS: Eight-week-old ApoE$^{-/-}$ mice were treated with *Lactobacillus rhamnosus* GR-1 daily for 12 weeks. ApoE$^{-/-}$ mice in the vehicle group and wild type (WT) mice were treated with normal saline. Serum lipid levels, histopathological analysis of the aorta, oxidative and inflammatory indexes and activation of the nuclear factor-kappa B (NF-κB) signaling pathway were examined.

RESULTS: Compared to ApoE$^{-/-}$ mice in the vehicle group, no changes in body weight or serum lipid levels were found in ApoE$^{-/-}$ mice treated with *Lactobacillus rhamnosus* GR-1. However, the administration of GR-1 slowed down the development of atherosclerosis and reduced plaque formation. Additionally, GR-1 attenuated the development of oxidative stress and chronic inflammation in a dose-dependent manner in ApoE$^{-/-}$ mice fed a high-fat diet. Furthermore, in ApoE$^{-/-}$ mice treated with GR-1, GR-1 was demonstrated to have a role in inhibiting the translocation of NF-κB p65 from the cytoplasm to the nucleus and suppressing the degradation of IκB-α.

CONCLUSIONS: We showed that the administration of GR-1 decreased atherosclerotic lesion size in ApoE$^{-/-}$ mice by reducing oxidative stress and inflammation. Additionally, the NF-κB signaling pathway might mediate these effects.

Key Words:
Atherosclerosis, Oxidative stress, Inflammation, ApoE$^{-/-}$ mice, *Lactobacillus rhamnosus* GR-1.

Introduction

Cardiovascular disease (CVD) and the underlying atherosclerotic progression are the major causes of mortality in both developed and developing countries[1]. Elevated serum cholesterol has been traditionally considered the important risk factor for CVD and atherosclerosis in humans[2]. Hjermann[3] revealed that a 1% decrease in serum cholesterol level will decrease the risk of coronary heart disease by 2%. However, other clinical studies[4] have demonstrated that even if a reduction in low-density lipoprotein (LDL) cholesterol is achieved, more than 50% of cardiovascular risk remains. In fact, inflammation and oxidative stress are now widely considered to play vital roles in atherosclerotic progression[5-7]. Thus, besides lipid-manipulation therapy, anti-inflammatory therapy and anti-oxidative stress treatments have now attracted the attention of many researchers[8-10]. Probiotic bacteria, which

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are defined as living microorganisms that can exert beneficial effects on the health of the host when administered adequately, have demonstrated many potential health effects in previous studies[11-14], including lowering serum cholesterol[15,16] as well as anti-inflammatory[17] and anti-oxidative stress activities[18]. Hence, the cholesterol-lowering, anti-inflammatory and anti-oxidative stress effects of probiotic bacteria may have a potential role in the prevention of atherogenesis. Gan et al[19] found that *Lactobacillus rhamnosus* GR-1 could attenuate the progression of heart failure post-infarction in rats. However, there are few studies about whether administration of *Lactobacillus rhamnosus* GR-1 will be beneficial for lowering the serum cholesterol level along with producing anti-inflammatory or anti-oxidative stress effects. In the present work, the major objectives were to investigate the cholesterol-lowering effects and the anti-inflammatory as well as antioxidant effects of *Lactobacillus rhamnosus* GR-1 in apolipoprotein-E knockout ($ApoE^{-/-}$) mice and to explore whether *Lactobacillus rhamnosus* GR-1 had a role in the prevention of atherogenesis.

Materials and Methods

Animals and Diet

Male $ApoE^{-/-}$ mice and C57BL/6J mice (8 week, 22–24 g) were obtained from the Institute Animal Research Laboratory at Nanjing University. All experimental procedures were approved by the Ethics Committee of Nanjing Hospital of Traditional Chinese Medicine. This study was conformed to the Institutional Guidelines for the Care and Use of Experimental Animals. The mice were randomly divided into four groups: (a) the wild type (WT) group, which included C57BL/6J mice orally treated with 0.5 mL normal saline daily; (b) the vehicle group, which included $ApoE^{-/-}$ mice orally treated with 0.5 mL normal saline daily; (c) the GR1. L group, which included $ApoE^{-/-}$ mice orally treated with a low dose (5×10^7 CFU) of *Lactobacillus rhamnosus* GR-1 daily; and (d) the GR1. H group, which included $ApoE^{-/-}$ mice orally treated with a high dose (5×10^8 CFU) of *Lactobacillus rhamnosus* GR-1 daily. The treatment course was 12 weeks. All of the $ApoE^{-/-}$ mice were fed a high-cholesterol and high-fat diet containing 0.25% cholesterol and 15% fat, whereas the WT group mice were fed a standard diet. Body weight was monitored once a week.

Bacteria

The resuscitation and subsequent propagation of *Lactobacillus rhamnosus* GR-1 were conducted as previously described in MRS broth (37°C, 18 hours, anaerobic conditions)[19]. The organisms then appeared on MRS agar (BD Difco, Franklin Lakes, NJ, USA). Single colonies were selected to inoculate 3 mL of MRS broth and 500 mL MRS broth. The strain was finally centrifuged at 1600 g for 20 minutes and was resuspended in 25 mL sterile skim milk (10% NFDM, Nestlé, Vevey, Switzerland) at final concentrations of 1×10^8 CFU/mL and 1×10^9 CFU/mL. GR-1 was administered orally to the $ApoE^{-/-}$ mice in the drinking water daily.

Sample Preparation

At the end of the treatment course, the mice in each group were sacrificed. A blood sample was collected through the retro-orbital plexus, and then the mice were perfused using ice-cold normal saline. The aorta, spleen and liver were collected and stored in liquid nitrogen before analysis.

Histopathological Analysis

The thoracic aorta samples were cut and fixed with 4% paraformaldehyde (Boster, Wuhan, China) and then embedded in an ice bag glued to a slide. Hematoxylin and eosin (HE) staining (Boster, Wuhan, China) of the transverse section of the aorta was used to compare the pathological changes in each group of mice.

Measurement of Serum Lipids

Serum levels of high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triglyceride (TG), glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), and direct conjugated bilirubin (DBIL) were determined with enzymatic colorimetric assays. (HITAC 7170 Automatic Analyzer, Tokyo, Japan).

Enzyme-Linked Immunosorbent Assay (ELISA) Assay

Serum oxygenized low-density lipoprotein (ox-LDL), malondialdehyde (MDA) and the activity of superoxide dismutase (SOD) were evaluated using ELISA kit (Cusabio Biology, Wuhan, China) according to the instructions. Serum tumor necrosis factor-α (TNF-α), monocyte chemotactic protein 1 (MCP-1) and interleukin-6 (IL-6) levels were

evaluated through the ELISA kit (Cusabio Biology, Wuhan, China) following the instructions.

Western Blot Analysis

Protein concentrations were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Western blotting was performed to identify protein expression levels of matrix metalloprotein-9 (MMP-9), matrix metalloprotein-2 (MMP-2), IκB-a, cytoplasm p65 and nuclear p65 as described by the manufacturers (Cusabio Biology, Wuhan, China).

Statistical Analysis

Continuous variables were represented as the means ± SD (standard deviation). For multiple comparisons, one-way ANOVA, followed by Post-Hoc Test (Least Significant Difference), was performed to analyze the data using Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS, Inc., Chicago, IL, USA), and p-values < 0.05 were considered statistically significant.

Results

Administration of GR-1 Slowed Down the Development of Atherosclerosis and Reduced Plaque Formation in ApoE$^{-/-}$ Mice Fed a High-Fat Diet

After 4 weeks of a high-fat diet, ApoE$^{-/-}$ mice were divided into 3 groups including the vehicle group (high-fat diet for 12 weeks), the GR-1. L group (high-fat diet with low concentration of GR-1 for 12 weeks) and the GR-1. H group (high-fat diet with high concentration of GR-1 for 12 weeks). To determine whether the administration of GR-1 slowed down the development of atherosclerosis and reduced plaque formation in ApoE$^{-/-}$ mice fed a high-fat diet, we used the HE staining of the transverse section of the aorta to compare the pathological changes of the aorta in each group of mice. In the WT group, we found that there was no plaque formation, and the endometrium was thin and smooth. The endothelial cells were arranged regularly, the thickness of the middle membrane was normal, and the smooth muscle cells were orderly arranged (Figure 1A). In the vehicle group, there were many plaques in the aorta accompanied by inflammatory cell aggregation and severe atrophy of the medial membrane. In addition, the structure of the blood vessel wall was not clear, there was irregular thickening of the inner membrane, formation of poroma, and the presence of lipid core and a large number of foam cells were observed (Figure 1B). Compared to the vehicle group, the pathological changes in the *La.L* group were not very significant, but the plaque in the *La.H* group was smaller and thinner, the thickness of the endometrium was smaller, and there was a decrease in foam cells and infiltration of inflammatory cells. Smooth muscle cells in the middle membrane were arranged neatly, but some of them were still crisscrossed (Figure 1C and 1D).

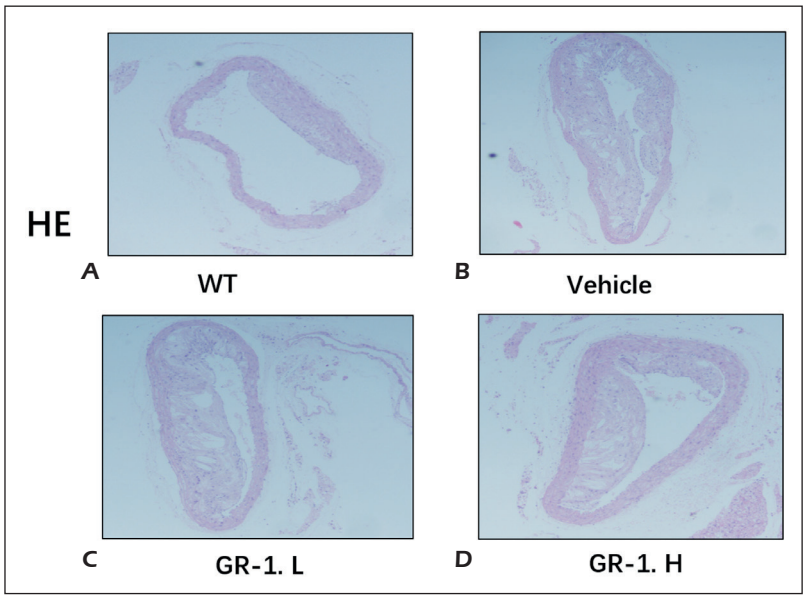

Figure 1. GR-1 slowed the development of atherosclerosis and reduced plaque formation. **A-D**, HE staining of transverse sections of the aorta was used to compare the pathological changes in the aorta for each group of mice (magnification: 10×).. All data are expressed as the mean ± SD (n=3).

Table I. Comparison of body weight, Viscera index, blood lipids, Glucose and liver function in each group of mice.

Variables	WT	ApoE$^{-/-}$vehicle	ApoE$^{-/-}$La.L	ApoE$^{-/-}$La.H
Body weight increment	132.8%±3.1%	152.3%±4.7%*	143.1%±3.8%*	148.4%±6.5%*
Liver index (%)	40.29±2.37	43.76±3.09*	43.09±3.2	43.01±2.67
Spleen index (%)	2.69±0.68	5.27±1.62**	4.42±0.87**#	4.16±0.76**#
HDL-C(mM)	1.25±0.15	1.27±0.29	1.18±0.23	1.16±.44
LDL-C(mM)	0.35±0.04	9.83±1.23***	9.26±0.96***	9.08±1.22***
TC (mM)	1.79±0.12	24.29±6.29***	25.41±6.89***	24.27±7.26***
TG (mM)	0.61±0.11	0.65±0.19	0.69±0.23	0.68±0.14
Glucose (mM)	6.62±2.27	8.72±2.97**	8.52±3.43**	7.08±3.29#
ALT (U/L)	42.19±9.28	92.39±10.34***	76.12±8.68***	64.68±8.2**#
AST (U/L)	201.13±51.28	223.28±39.23*	209.28±42.83	195.29±34.03#
TBIL (μM)	2.49±0.29	2.89±1.17*	2.67±0.91	2.42±1.07#
DBIL (μM)	1.08±0.28	1.02±0.39	0.98±0.23	0.99±0.31

*p<0.05, **p<0.001, ***p<0.001 *vs.* WT, #p<0.05 *vs.* vehicle.

Comparison of Visceral Index, Blood Lipids, Glucose and Liver Function Indexes of Mice in Each Group

As shown in Table I, the body weight increment index, liver index, LDL-C and TC were significantly lower in the WT group mice compared to the other 3 groups, but there were no significant differences among the other 3 groups. The spleen index, glucose, ALT and AST in vehicle group mice were significantly higher compared to the WT group and decreased markedly after treatment with GR-1. However, there was no difference in TG, TBIL and HDL-C among mice between the groups. The results demonstrated that GR-1 could improve spleen index and liver function as well as reduce blood glucose levels in ApoE$^{-/-}$ mice fed a high-fat diet, whereas there was no effect on blood lipids.

Effects of GR-1 on Oxidative Stress and Inflammatory Factors in ApoE$^{-/-}$ Mice Fed a High-Fat Diet

To investigate the roles of GR-1 on oxidative stress and inflammatory factors in ApoE$^{-/-}$ mice fed a high-fat diet, we used oral gavage to treat ApoE$^{-/-}$ mice fed a high-fat diet with either low or high concentrations of GR-1 for 12 weeks. As shown in Figure 2A-C, the oxidative stress index of oxLDL and MDA was significantly activated in ApoE$^{-/-}$ mice fed with a high-fat diet compared to the WT group mice, and GR-1 could markedly reduce the values of oxLDL and MDA in a concentration-dependent manner. In addition, ELISA analysis was used to measure the expression of inflammation cytokines TNF-α, MCP-1 and IL-6. As shown in Figure 2D-F, TNF-α, MCP-1 and IL-6 were significantly overexpressed in ApoE$^{-/-}$ mice fed with a high-fat diet, whereas the effect was reversed by GR-1 treatment, and the inflammatory factors decreased significantly after 12 weeks of oral gavage with GR-1. These results indicated that administration of a probiotic attenuates the development of oxidative stress and chronic inflammation in ApoE$^{-/-}$ mice fed a high-fat diet.

Administration of GR-1 Inhibits the Protein Expression of MMP-9 and MMP-2

Given that MMPs play a critical role in the pathogenesis of vulnerable plaques, we investigated whether administration of GR-1 in ApoE$^{-/-}$ mice fed a high-fat diet regulates the protein expression levels of MMP-9 and MMP-2. The results demonstrated that GR-1 treatment significantly reduced the protein expression of MMP-2 and MMP-9 (Figure 3A and 3B). This outcome suggested that administration of a probiotic may exert potential function to increase atherosclerotic plaque stability by inhibiting MMP-2 and MMP-9 expression.

Effect of GR-1 on the NF-ÐB Signaling Pathway

To investigate the effect of GR-1 on the NF-κB signaling pathway, we performed a Western blot analysis to evaluate the expression of IκB-a, cytoplasm p65 and nuclear p65 in each group of mice. Compared to WT mice, the protein expression of IκB-a and cytoplasmic p65 in ApoE$^{-/-}$ mice

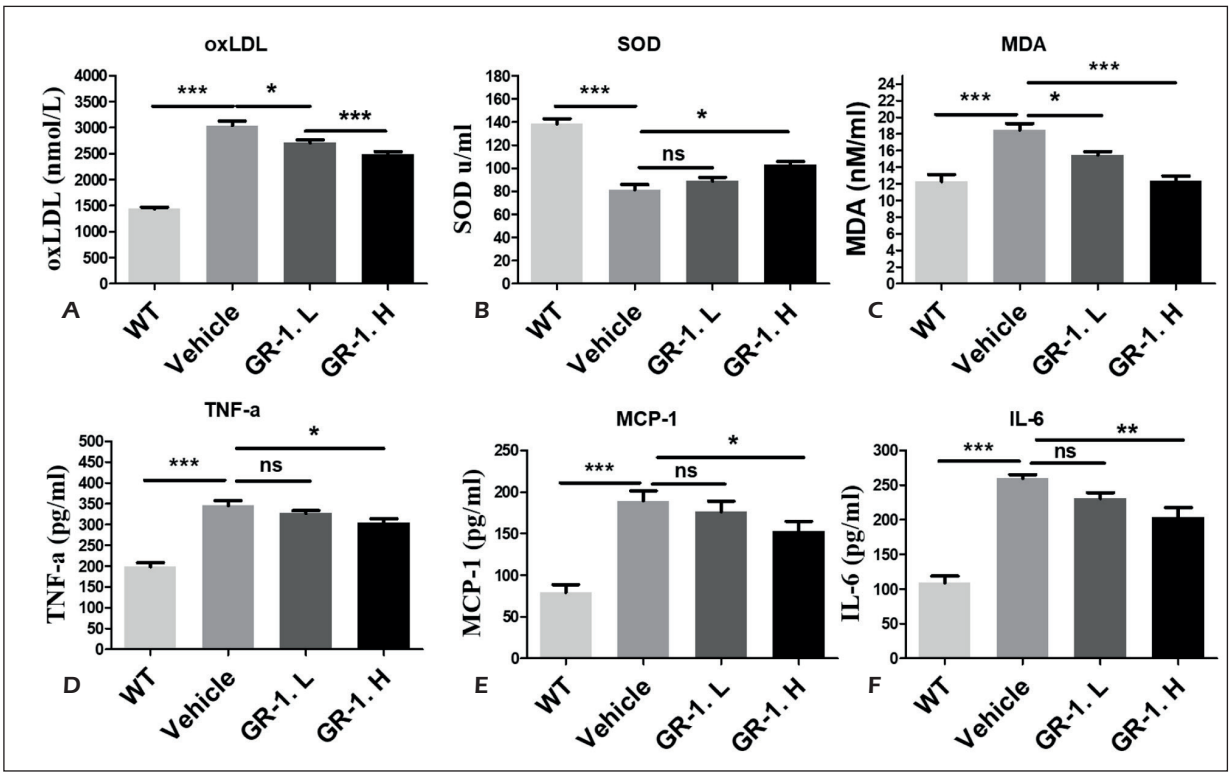

Figure 2. Effects of GR-1 on oxidative stress and inflammatory factors in ApoE-/- mice fed a high-fat diet. **A**, ELISA assay was used to detect the oxidative stress index of oxLDL. **B**-**C**, SOD and MDA kits were used to detect the oxidative stress index of SOD and MDA, respectively. **D**-**F**, ELISA analysis was used to detect the inflammatory cytokines TNF-a, MCP-1 and IL-6. All data are expressed as the mean ± SD (n=3). Each value of *p<0.05, **p<0.01, ***p<0.001 was deemed to indicate significant differences.

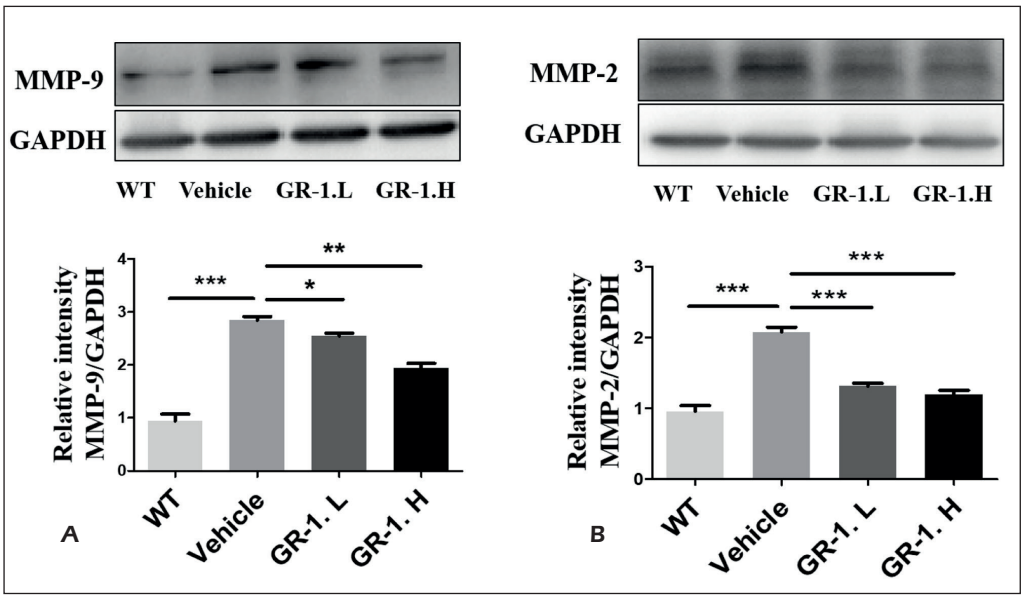

Figure 3. Administration of GR-1 inhibits the protein expression of MMP-9 and MMP-2. **A**-**B**, Western blot analysis was performed to examine the protein expression of MMP-9 and MMP-2 in each group of mice. All data are expressed as the mean ± SD (n=3). Each value of *p<0.05, **p<0.01, ***p<0.001 was deemed to indicate significant differences.

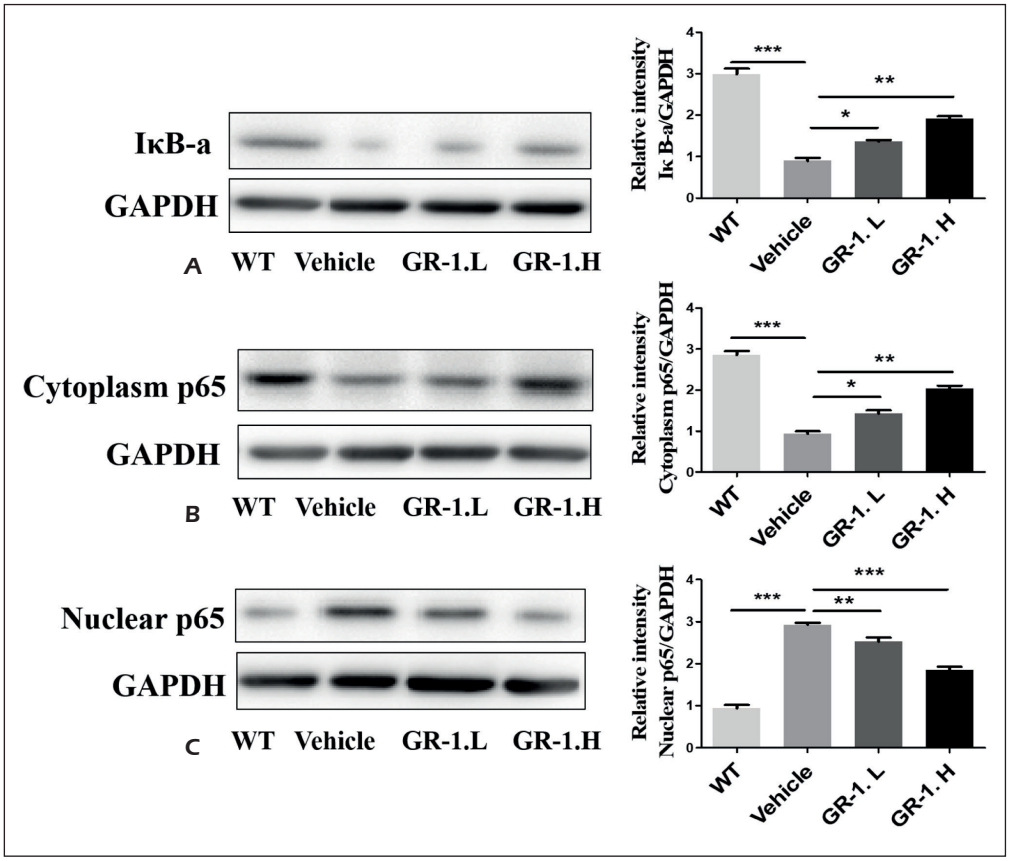

Figure 4. Effect of GR-1 on the NF-κB signaling pathway. **A-C**, Western blot analysis was used to examine the protein expression of IκB-a, cytoplasmic p65 and nuclear p65 in each group of mice. All data are expressed as the mean ± SD (n=3). Each value of *p<0.05, **p<0.01, ***p<0.001 was deemed to indicate significant differences.

fed with a high-fat diet was decreased, and the expression of IκB-a and cytoplasmic p65 in aortic tissues was upregulated after administration of GR-1 (Figure 4A and 4B). In addition, the protein expression of nuclear p65 was overexpressed in the vehicle group compared to the WT group of mice, whereas the expression of nuclear p65 was suppressed after administration of GR-1 in a concentration-dependent manner (Figure 4C). The results indicated that the NF-κB signaling pathway may be involved in the process of GR-1 inhibiting plaque formation.

Discussion

We demonstrated that administration of *Lactobacillus rhamnosus* GR-1 in ApoE$^{-/-}$ mice could attenuate the formation of atherosclerotic lesions, suppress oxidative stress by regulating the expression levels of oxLDL, SOD and MDA, and inhibit inflammation by modulating the levels of TNF-α, MCP-1 and IL-6. These effects may occur *via* inhibition of the NF-κB signaling pathway. However, the administration of GR-1 had no effect on serum lipid levels in ApoE$^{-/-}$ mice, suggesting that GR-1 had an anti-atherosclerotic role without affecting the levels of lipids. Atherosclerosis is the basis of cardiovascular diseases, such as hypertension and coronary heart disease and has caused serious harm to human health and national economies[20]. However, the specific causes and mechanisms of atherosclerosis have not yet been fully elucidated[20]. Elevated serum cholesterol, inflammation and oxidative stress are now considered important risk factors of atherosclerosis (As) in humans[2,5-7]. Previously scholars have shown that some kinds of probiotic bacteria could lower serum cholesterol levels. For example, Jones et al[15] have shown that consumption of BSH-active *L. reuteri* NCIMB 30242 could lower LDL-C, TC, apoB-100 and non-HDL-C in hypercholesterolemic humans. Lee et al[16] have demonstrated that the administration of *B. longum* SPM1207 could

help to manage hypercholesterolemia. In the present study, compared to ApoE$^{-/-}$ mice in the vehicle group, no differences were found in the serum levels of LDL-C TC or HDL in the *La.L* group and the *La.H* group, suggesting that GR-1 had no effect on serum lipid levels in ApoE$^{-/-}$ mice. However, HE staining of the transverse section of the aorta showed that a high concentration of GR-1 could reduce the formation of atherosclerotic plaques, which suggests GR-1 could attenuate atherosclerotic progression *via* other mechanisms instead of affecting lipid levels. As inflammation and oxidative stress are one of the factors contributing to the formation of atherosclerosis[5-7], we hypothesized that GR-1 may influence atherosclerosis through these two mechanisms. Interestingly, previous studies have shown some kinds of probiotic bacteria could play a role in anti-inflammation and anti-oxidative stress activities. Archer et al[17] found that *L. fermentum* MCC 2759, *L. fermentum* MCC 2760 and *L. delbrueckii* MCC 2775 exhibited anti-inflammatory abilities in an acute inflammatory paw edema model, while Yadav et al[18] demonstrated that *Lactobacillus fermentum* MTCC 5898 could attenuate oxidative stress and inflammation in rats fed a cholesterol-enriched diet. Oxidation is a part of the normal organism's life activities. It can provide energy for the body's activities, but excessive oxidation can cause damage to the organism[21]. Ox-LDL is the product of LDL oxidation and has a variety of bioactivities that can inhibit the activity of eNOS and the formation of NO, which results in vascular systolic dysfunction, promotes the proliferation and migration of endothelial and smooth muscle cells to accelerate the formation of atherosclerosis, increases the instability of atherosclerotic plaques and causes plaque rupture[22-24]. SODs are enzymes that can catalyze the dismutation of the superoxide radical anion (O_2^-) effectively[25]. Overexpressing SOD enzyme could decrease the atherosclerotic plaque formation area in ApoE$^{-/-}$ mice[26]. MDA is one of the final products of lipid peroxidation, so it is commonly used to reflect the degree of lipid peroxidation[27]. In a previous study, we showed that GR1 could reduce serum oxLDL levels in ApoE$^{-/-}$ mice. Additionally, the administration of GR1 could increase the serum levels of SOD and decrease the content of MDA. These results suggested that GR1 has an action of anti-oxidation in ApoE$^{-/-}$ mice fed a high-fat diet. TNF-α is a well-known multifunctional cytokine that has been identified throughout atherosclerotic progression[28]. In atherosclerosis progression, the potential roles for TNF-α include recruiting inflammatory cells[29], promoting the poor remodeling of vascular smooth muscle cells[30, 31], and acting as a pro-inflammatory factor in plaque rupture[30]. Indeed, Ohta *et al*[32] found that the atherosclerotic plaque area in ApoE$^{-/-}$/TNF-α$^{-/-}$ mice was significantly smaller than that in ApoE$^{-/-}$ mice without affecting changes in serum cholesterol levels. MCP-1 is mainly expressed by endothelial cells and inflammatory cells and has been reported to play a vital role in the pathogenesis for atherosclerosis[33]. IL-6 has been identified as an independent risk factor for carotid atherosclerosis[34]. Additionally, the administration of IL-6 can enlarge atherosclerotic lesions in ApoE$^{-/-}$ mice[35]. In our study, TNF-α, MCP-1 and IL-6 expressions were significantly overexpressed in ApoE$^{-/-}$ mice fed a high-fat diet, whereas the effect was reversed after 12 weeks of oral gavage treatment with GR-1, indicating that the administration of GR1 could attenuate the development of chronic inflammation in ApoE$^{-/-}$ mice fed a high-fat diet. MMP-2 and MMP-9 have been studied thoroughly in vascular disease[36]. Many investigations[37,38] have demonstrated that circulating MMP-2 and MMP-9 levels increase in atherosclerosis and are related to plaque instability. Additionally, MMP-2 and MMP-9 are involved in fibrous cap formation, collagen deposition and calcification[36]. We showed that GR-1 significantly reduced the protein expression levels of MMP-2 and MMP-9, which suggests that administration of GR-1 may have a role in increasing atherosclerotic plaque stability by inhibiting MMP-2 and MMP-9. NF-κB is a transcription factor that is involved in certain inflammatory diseases, including atherosclerosis[39]. The NF-κB family consists of the members' p50, p52, p65 (RelA), c-Rel, and RelB, which form various homo- and heterodimers[40]. Detecting the expression of p65 can represent the activation state of NF-κB[41]. The IκB proteins are regulatory proteins that bind to NF-κB and prevent its nuclear translocation and transcriptional activity[39]. When stimulated by factors, such as cytokines, protein kinase C activators and oxidants, the phosphorylation of IκB loses its inhibitory effect on NF-κB, and NF-κB is activated[39]. Then, NF-κB can translocate into the nucleus to activate the transcription of target genes, including proinflammatory cytokines, adhesion molecules, chemokines and so on[39]. On the other hand, the genes regulated by NF-κB, such as TNF-α and IL-1β, can further induce the activation of NF-κB, forming a positive feedback effect to enhance

the inflammatory response[39]. In a previous study, the expression levels of IκB-a and cytoplasm p65 that decreased in ApoE$^{-/-}$ mice fed with a high-fat diet were upregulated after administration of GR-1. In addition, the expression of nuclear p65 was suppressed after administration of GR-1 in a concentration-dependent manner, which indicates that NF-κB signaling pathway may be involved in the process of GR-1 inhibiting plaque formation.

Conclusions

We demonstrated that administration of GR-1 decreased atherosclerotic lesion size in ApoE$^{-/-}$ mice by reducing oxidative stress and inflammatory states. Additionally, the NF-κB signaling pathway might take part in these effects.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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