Associations of CXCL16, miR-146a and miR-146b in atherosclerotic apolipoprotein E-knockout mice

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Abstract. Atherosclerosis is the primary cause of cardiovascular and cerebrovascular diseases. Recent studies have revealed that C-X-C motif chemokine ligand 16 (CXCL16), microRNA (miR)-146a and miR-146b may have important roles in atherosclerotic diseases. However, the associations of CXCL16, miR-146a and miR-146b in atherosclerotic diseases *in vivo* remain unclear. Previous studies have demonstrated that miR-146a and miR-146b may negatively regulate the toll like receptor (TLR4)/nuclear factor (NF)-κB signaling pathway to repress the inflammatory response. The present study investigated the associations of CXCL16, miR-146a and miR-146b in atherosclerotic apolipoprotein E (ApoE)-/- mice *in vivo*. The expression levels of CXCL16, TLR4/NF-κB signaling pathway, miR-146a and miR-146b in the control and atherosclerotic ApoE-/- mice were investigated via reverse transcription-quantitative polymerase chain reaction and western blot analysis. The present study demonstrated that the expression of CXCL16 was significantly upregulated in atherosclerotic ApoE-/- mice compared with control ApoE-/- mice. The expression levels of TRL4, interleukin-1 receptor-associated kinase 1, tumor necrosis factor receptor associated factor 6, NF-κB, tumor necrosis factor-α and interleukin-1β were also significantly upregulated in atherosclerotic ApoE-/- mice compared with control mice. However, the present study revealed that the expression levels of miR-146a and miR-146b were significantly downregulated in atherosclerotic ApoE-/- mice compared with control ApoE-/- mice. Overall, the results of the present study suggested that CXCL16 may regulate the TRL4/NF-κB/CXCL16 signaling pathway, and that miR-146a and miR-146b may negatively regulate CXCL16 via this pathway in atherosclerosis *in vivo*.

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Introduction

Atherosclerosis can lead to vascular stenosis, thrombosis and vascular occlusion, which are characterized by chronic inflammatory disease and can cause ischemic damage to vital organs (1,2). During the development of atherosclerosis, numerous inflammatory cells and cytokines are mediated by specific receptors, intracellular signal transduction or gene transfer modifications, which can affect functional protein expression and the development of inflammatory responses (3). Adhesion molecules are considered to represent important factors in the initiation of inflammatory reactions associated with atherosclerosis, and chemokines have important roles in linking inflammation and atherosclerosis (4).

The expression levels of the previously discovered C-X-C motif chemokine ligand 16 (CXCL16) are significantly increased in atherosclerosis, and CXCL16 is involved in the occurrence of inflammation and atherosclerosis (5,6). CXCL16 is produced by numerous inflammatory cells and is preferentially expressed within atherosclerotic plaques, including macrophages, vascular endothelial cell and smooth muscle cells, and possesses the functions of chemokines, adhesion molecules and scavenger receptors (7,8). Previous studies have demonstrated that increased serum CXCL16 levels represent an independent risk factor in ischemic stroke with atherosclerosis, and may also represent a novel biomarker for the prediction of ischemic stroke incidence (9,10). Studies have revealed that CXCL16 expression levels in atherosclerotic lesions are markedly increased following induction with bacterial lipopolysaccharide (LPS), endotoxin and nuclear factor (NF)-κB, which improves the absorption capacity of oxidized low-density lipoprotein and aggravates the development of atherosclerosis (11,12).

Toll like receptors (TLRs) have important roles in the initiation of inflammatory responses (13). LPS is a common inflammatory stimulus of cells, and can be recognized by TLR4. Myeloid differentiation factor 88 functions as an important link between TLR4 and interleukin-1 receptor

associated kinase 1 (IRAK1), which binds with tumor necrosis factor receptor-associated factor 6 (TRAF6) and subsequently activates downstream NF-κB, which results in an increased expression of immune response genes (14). The NF-κB pathway is activated by TLR4 and represents the main signaling pathway associated with the regulation of inflammatory responses, as well as an intermediate link in LPS-induced increased CXCL16 expression (15). Thus, it can be suggested that NF-κB has an important role in the development of inflammation. LPS is recognized by TRL4, resulting in NF-κB activation. Activated NF-κB subsequently triggers target gene transcription and inflammatory responses by inducing CXCL16, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and other inflammatory factors, which promote the formation and progression of atherosclerotic plaques (11). Therefore, the inhibition of TRL4/NF-κB/CXCL16 pathway and the suppression of inflammatory reactions may represent novel therapeutic targets for the treatment of patients with atherosclerotic diseases.

Long-term or exaggerated inflammatory responses are harmful, and it is therefore important to regulate cellular negative feedback loops to suppress or inhibit the inflammatory response (16). Micro (mi)RNAs are small, noncoding RNA molecules of 21-24 nucleotides in length that function as negative regulators to mediate complex biological reactions via binding to the 3'-untranslated region (UTR) of mRNAs, which subsequently inhibits the translation or affects the stability of the target gene (17,18). miR-146a was the first miRNA revealed to be associated with the inflammatory response, and is rapidly induced by inflammatory stimulation (19). Studies have demonstrated that miR-146a has an important negative effect on the inflammatory response (20-22). miR-146b is an additional member of the miR-146 family, only differing from miR-146a by 2 nucleotides at the 3'UTR in its mature sequence (23). It has been demonstrated that miR-146a is a NF-κB-dependent gene that can activate innate immune signaling pathways (19). miR-146a targets adaptor proteins of TRAF6 and IRAK1, and subsequently inhibits NF-κB activation, which suggests that miR-146a represents a negative feedback mechanism for the regulation of the NF-κB pathway in monocytes (24,25). It has been previously revealed that miR-146a inhibits the expression levels of proteins associated with the NF-κB pathway, such as IL-6, IL-8 and TNF-α (19,26). miR-146b is an IL-10 reactive miRNA, which ameliorates inflammatory reactions by targeting the TLR4 pathway (23). Associations between miR-146b and the TLR4/NF-κB signaling pathway remain unclear. One study demonstrated that miR-146b may mediate the TLR4 signaling pathway via direct regulation of numerous proteins, such as TLR4, IRAK1 and TRAF6; rather than via the NF-κB signaling pathway (23). The same study also revealed that elevated miR-146b expression may lead to a marked reduction in the LPS-dependent production of several inflammatory cytokines, such as IL-6, TNF-α and CXCL10 (23). However, a further study demonstrated that miR-146a and miR-146b regulate apoptosis and inflammatory cytokines in human dendritic cells via regulation of the TRAF6/IRAK1/NF-κB pathway (27). Therefore, it is important to investigate the associations between CXCL16, the NF-kB pathway, miR-146a and miR-146b.

To the best of the author's knowledge, there are no reports regarding the associations of CXCL16, miR-146a and miR-146b in atherosclerotic disease *in vivo*. The present study investigated the associations between CXCL16, miR-146a and miR146b in atherosclerotic disease *in vivo*. Atherosclerotic mouse models were established, in which a perivascular collar was placed around the carotid artery of ApoE-/- mice to form atherosclerotic vascular lesions. The results of the present study may further the understanding of the mechanisms underlying the associations between CXCL16, miR-146a, miR-146b and the TLR4/NF-κB signaling pathway during inflammatory responses associated with atherosclerosis.

Materials and methods

Mouse experiments. A total of 24 male ApoE-/- C57BL/6J mice (8-week-old; weight 18-22 g) were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). All mice were housed under a 12-h light/dark cycle at a room temperature of 22˚C and 50-60% relative humidity. All mice had free access to food and water. The study protocol was approved by the Animal Ethics Committee of Qingdao University prior to experimentation.

All mice were randomly divided into two groups (n=12 per group): A control group and a model group. In the first week, all mice were fed with normal food. However, from the second week onwards, the ApoE-/- mice in the model group were fed a high-fat diet, which constituted 15% cocoa butter, 0.25% cholesterol and normal food (28,29). The ApoE-/- mice in the control group remained on a diet of normal food. From the fifteenth day onwards, each mouse in the model group had a perivascular collar placed around the right common carotid artery (28-30). Each of the mice in the control group underwent sham surgery. A total of 8 weeks post-surgery, all mice were sacrificed and the blood and right common carotid arteries were collected for the subsequent experiments (28).

Lipid analysis. Levels of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) were determined in the blood gathered from the femoral artery of the mice. The lipid levels were measured by an Olympus AU640 automatic biochemical analyzer (Olympus Corporation, Tokyo, Japan) at the clinical laboratory of The Affiliated Hospital of Qingdao University.

Histopathological analysis. The common carotid artery were fixed in 10% neutral formalin at 4˚C for 24 h, dehydrated and then embedded in paraffin. Continuous transverse paraffin sections of 5 μm were obtained. Selective staining was performed with hematoxylin and eosin (HE) at 50-μm intervals at 25˚C for 20 min. A pathological image analyzer was used to measure the size of the staining patch area. Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to determine the proportion of atherosclerotic plaque area to the luminal area (31,32).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from the right common carotid arteries using TRIzol® (Thermo

Fisher Scientific, Inc., Waltham, MA, USA). The purity and concentration of the total RNA were determined using a spectrophotometer, and samples exhibiting a range of A260/280 between 1.8 and 2.0 were regarded suitable for further experimentation. RT-qPCR was used to determine the expression levels of miR-146a, miR-146b, TLR4, IRAK1, TRAF6, NF-κB, CXCL16, TNF-α and IL-1β. A PrimeScript TM miRNA qPCR Starter Kit Ver.2.0 (Takara Bio, Inc., Otsu, Japan) was used. Primer sequences used for qPCR of miRNAs were: miR-146a-5p: 5'-GCCGTGAGAACTGAATTCCATG-3'; miR-146b-5p: 5'-GAGCTGAGAACTGAATTCCATAG-3'; the internal reference of miR-146a and miR-146b was U6 (Takara Bio, Inc.). The primer sequences for U6 was: Forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The primer sequences used for qPCR of proteins were: TLR4 forward, 5'-TCAGAGCCGTTGGTGTATCTT-3' and reverse, 5'-TGTCCTCCCATTCCAGGTAG-3'; IRAK1 forward, 5'-ATAAGGCAGGCAATGTGAGG-3' and reverse, 5'-TCATACCCACTGAGCCATCTC-3'; TRAF6 forward, 5'-TCGGAGTGCCGTGTATGTAG-3' and reverse, 5'-CACCTTCTTCTGGCTTTCGT-3'; NF-κB forward, 5'-TGGACGACTCTTGGGAGAAG-3' and reverse, 5'-CACAGGCTCATACGGTTTCC-3'; CXCL16 forward, 5'-CAGGCTCGTCTCCATCAGT-3' and reverse, 5'-GTAGAGGCAAAGGGTCAGCA-3'; TNF-α forward, 5'-TCTGGGCAGGTCTACTTTGG-3' and reverse, 5'-GGTTGAGGGTGTCTGAAGGA-3'; IL-1β forward, 5'-CATCAGCACCTCTCAAGCAG-3' and reverse, 5'-AGTCCACATTCAGCACAGGA-3'. The relative expression levels were normalized to the internal reference gene GAPDH. The primer sequences of GAPDH were: Forward, 5'-AACAGCCTCAAGATCATCAGCAA-3' and reverse, 5'-GACTGTGGTCATGAGTCCTTCCA-3'.

The reaction conditions for miR-146a and miR-146b were: Initial denaturation step for 30 sec at 95˚C, denaturing at 95˚C for 10 sec, annealing and elongation 30 sec at 60˚C for 45 cycles. The reaction conditions of TRL4, IRAK1, TRAF6, NF-kB and CXCL16 were: Initial denaturation step for 30 sec at 95˚C, denaturing at 95˚C for 10 sec, annealing 20 sec at 55˚C and elongation for 30 sec at 72˚C for 55 cycles. The experiments were repeated under the above experimental conditions 3 times. The $2^{-\Delta\Delta Cq}$ method was used to compare the relative expression results (33).

Western blot analysis. Total protein was extracted from the right common carotid artery in each group using a mixture of tissue lysate and protease inhibitor (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China; 1:100), and the bicinchoninic acid method was used to determine total protein concentration. Then, 20 μg protein/lane was separated via SDS-PAGE at the following percentages: IRAK (6%), IL-1β (12%), TNF-α (12%), CXCL16 (12%), TRAF6 (8%), TLR4 (8%) and NF-κB (8%). The separated proteins were subsequently transferred onto a polyvinylidene difluoride membrane (0.2 μm for IL-1β and TNF-α and 0.45 μm for the other proteins). The membranes were blocked with 5% skimmed milk (Beijing Solarbio Science & Technology Co., Ltd.) at 4˚C for 2 h. The Primary antibodies against the following proteins were used: β-actin (cat. no. BM0627; 1:200; Wuhan Boster Biological Technology, Ltd., Wuhan, China), TLR4 (cat. no. ab13867; 1:250; Abcam, Cambridge, UK), IRAK1 (cat. no. 4504; 1:1,000; CST Biological Reagents Co., Ltd., Shanghai, China), TRAF6 (cat. no. ab33915; 1:6,000; Abcam), NF-κB (cat. no. 8242; 1:2,000; CST Biological Reagents Co., Ltd.), CXCL16 (cat. no. ab119350; 1:1,000; Abcam), TNF-α (cat. no. 3707; 1:1,000; CST Biological Reagents Co., Ltd.) and IL-1β (cat. no. 12242; 1:1,000; CST Biological Reagents Co., Ltd.). The membranes were washed in TBST buffer (Beijing Solarbio Science & Technology Co., Ltd.) 4 times for 8 min at a time at 25˚C and then incubated at 4˚C for 1 h with Goat anti-Mouse IgG (cat. no. BA1050; 1:3,500; Wuhan Boster Biological Technology, Ltd.) and Goat anti-Rabbit IgG (cat. no. 1:1,500; BA1054; Wuhan Boster Biological Technology, Ltd.). Protein bands were visualized using an ECL kit (Wuhan Boster Biological Technology, Ltd.). Fusion FX7 (Vilber Lourmat, Marne-la-Vallée, France) was used to acquire images, and the results were analyzed with ImageJ version 1.38 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All experiments were performed in triplicate and SPSS software version 19.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. All data are expressed as the mean ± standard deviation. Statistically significant differences between the two groups were determined using the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Body weight and lipid levels of ApoE-/- mice. No significant differences in body weight were observed between the two groups on the 1 day and 70 day time intervals ($P>0.05$; Table I). Levels of TG, TC and LDL-c in the model group were significantly increased compared with the control group ($P<0.05$; Table I); however, no significant differences were observed between the model and control groups regarding HDL-c levels ($P>0.05$; Table I).

Pathological analysis of common carotid artery tissues obtained from ApoE-/- mice. H&E staining results revealed that there were no marked pathological symptoms were observed in the vascular lumen and intima of ApoE-/- mice belonging to the control group (Fig. 1A); however, significant plaques and lumen stenosis were observed in ApoE-/- mice belonging to the model group (Fig. 1B). In addition, the ratio of the lesion volume to the vessel volume was significantly increased in the model group compared with the control group ($P<0.01$; Fig. 1C). Therefore, the results demonstrated that ApoE-/- mice in the model group exhibited significantly increased formation of atherosclerotic plaques compared with ApoE-/- mice in the control group.

Expression levels of CXCL16 and proteins associated with the TRL4/NF-κB signaling pathway are increased in atherosclerotic ApoE-/- mice. The CXCL16 and TRL4/NF-κB signaling pathway serve important roles in the inflammatory responses, and so their expression in atherosclerotic ApoE-/- mice was investigated using RT-qPCR and western blot analysis in the present study. The results revealed that the mRNA and protein

Table I. Body weight and lipid levels of ApoE-/- mice.

	Body weight at the 1 day time interval (g)	Body weight at the 70 day time interval (g)	TC (mmol/l)	TG (mmol/l)	LDL-c (mmol/l)	HDL-c (mmol/l)
Control group	19.20±1.30	27.75±1.89	13.06±1.72	2.19±0.11	1.03±0.12	1.77±0.37
Model group	21.00±1.00	28.67±0.58	24.72±2.30[a]	2.73±0.23[a]	4.62±0.14[a]	1.34±0.18
P-value	0.066	0.073	0.002	0.022	<0.0001	0.105

[a]P<0.05 vs. control group. TC, total cholesterol; TG, triglycerides; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol.

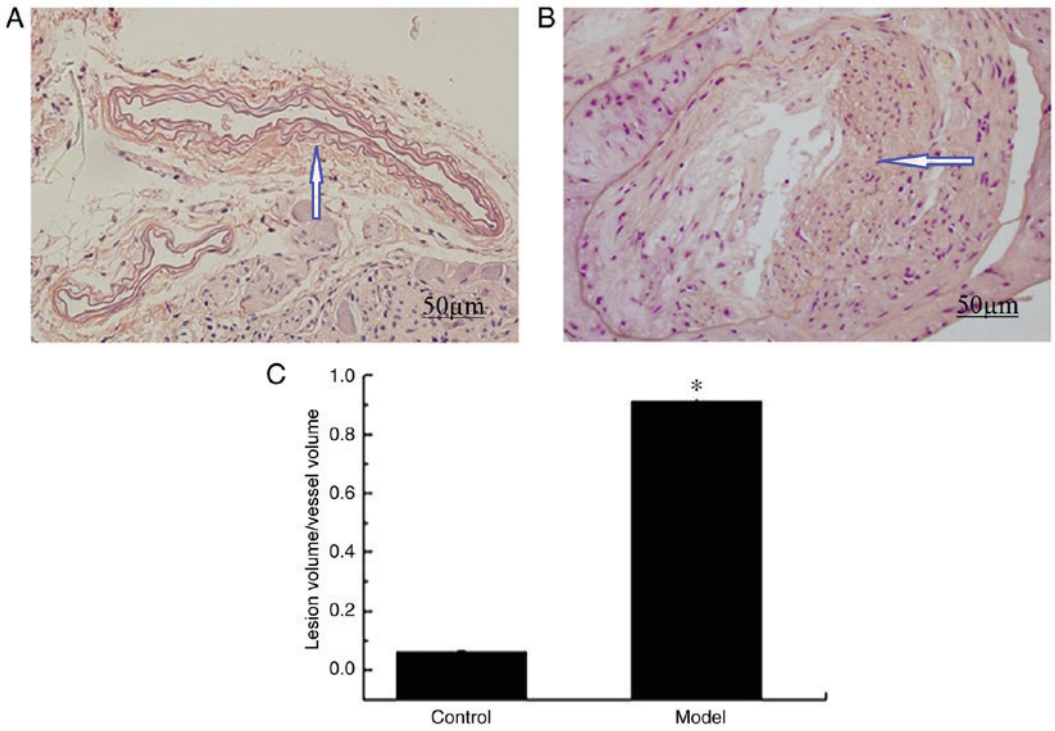

Figure 1. Pathological analysis of common carotid artery tissues obtained from ApoE-/- mice. (A) No pathological symptoms were observed in the vascular lumen of tissues obtained from ApoE-/- mice in the control group were observed, and the tunicae intima vasorum remained smooth and was not destroyed (magnification, x400; n=6). (B) In ApoE-/- mice belonging to the model group, the vascular intimal integrity was revealed to be damaged and thickened, a large quantity of foam cells and inflammatory cells under the damaged endometrium was observed and the vascular lumen was markedly narrowed (arrowed; magnification, x400; n=6). scale bars=50 μm. (C) The ratio of lesion volume to vessel volume was significantly increased in the model group compared with the control group. *P<0.01 vs. control group. ApoE-/-, apolipoprotein E-knockout.

levels of CXCL16 were significantly upregulated in the model group compared with the control group (P<0.01; Fig. 2). The results also demonstrated that the mRNA and protein levels of TRL4, IRAK1, TRAF6, NF-κB, TNF-α and IL-1β were significantly upregulated in the model group compared with the control group (P<0.05 and P<0.01; Fig. 2). These results revealed that the expression levels of CXCL16 and proteins associated with the TRL4/NF-κB signaling pathway were significantly upregulated in atherosclerotic ApoE-/- mice compared with control ApoE-/- mice.

Expression levels of miR-146a and miR-146b are decreased in atherosclerotic ApoE-/- mice. To investigate the expression levels of miR-146a and miR146b in atherosclerotic disease, ApoE-/- mice were fitted with a perivascular collar and administered a high-fat diet. The expression levels of miR-146a and miR-146b were investigated using RT-qPCR. Compared with the control group, the expression levels of miR-146a and miR-146b were significantly suppressed in the ApoE-/- mice belonging to the model group compared with ApoE-/- mice belonging to the control group (P<0.05 and P<0.01; Fig. 3).

Discussion

In the present study, a perivascular collar was placed around the carotid artery of ApoE-/- mice to induce atherosclerotic pathological changes, as previously described (29,30,34). The results of the present study revealed a significant formation of atherosclerotic plaques in the carotid artery of atherosclerotic ApoE-/- mice compared with the control ApoE-/- mice. Recent studies have demonstrated that CXCL16 expression is significantly increased in patients suffering from acute ischemic

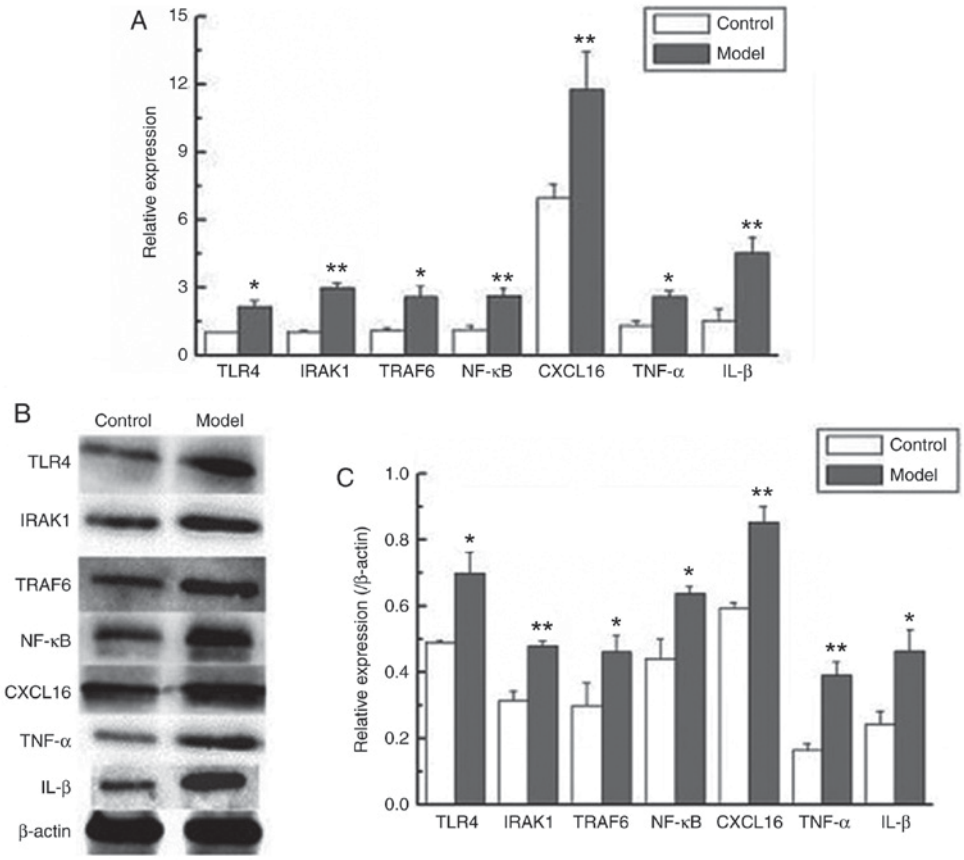

Figure 2. Protein and mRNA expression levels of CXCL16 and proteins associated with the TRL4/NF-κB signaling pathway in ApoE-/- mice. (A) mRNA expression levels of CXCL16 and proteins associated with the TRL4/NF-κB signaling pathway in ApoE-/- mice were investigated using reverse transcription-quantitative polymerase chain reaction. (B) Western blotting was used to investigate the protein levels of CXCL16 and proteins associated with the TRL4/NF-κB signaling pathway in ApoE-/- mice, and (C) quantification of western blotting results was performed. Data are presented as the mean ± standard deviation (n=6). *P<0.05 and **P<0.01 vs. control group. mRNA, messenger RNA; NF-κB, nuclear factor-κB; CXCL16, C-X-C motif chemokine ligand 16; TLR4, toll like receptor 4; IRAK1, interleukin-1 receptor associated kinase 1; TRAF6, tumor necrosis factor receptor-associated factor 6; TNF-α, tumor necrosis factor α; IL-β, interleukin-β.

stroke, and that enhanced CXCL16 levels may represent a biomarker for ischemic stroke incidence prediction (9,10). The present study demonstrated that the mRNA and protein levels of CXCL16 were significantly upregulated in the model group exhibiting atherosclerotic plaques. Previous research has demonstrated that the level of CXCL16 increases in atherosclerotic ApoE-/- mice and may represent a potential atherogenic biomarker (35). In studies of coronary heart disease (CHD), a high level of CXCL16 has been revealed to be associated with the severity of acute coronary syndrome, and may represent a potential biomarker for epidemiological and clinical application in CHD (36,37). Laugsand *et al* (38) demonstrated that CXCL16 is closely associated with the risk of myocardial infarction and may aid in assessing cardiovascular risk. These studies have revealed that CXCL16 is involved in atherosclerotic disease. However, the pathophysiological role of CXCL16 in atherosclerotic diseases *in vivo* remains unclear.

Furthermore, the present study demonstrated that the expression of NF-κB, TNF-α and IL-1β were significantly increased in atherosclerotic ApoE-/- mice compared with control ApoE-/- mice. NF-κB is activated by TLR4, which is associated with inflammation (39,40). TNF-α and IL-1β are located downstream of the NF-κB pathway and are activated by NF-κB (41,42). In an *in vitro* study, simulated cardiac ischemia/reperfusion injury in human umbilical vein endothelial cells enhanced levels of CXCL16, TNF-α and intercellular adhesion molecule-1 (ICAM-1), thus suggesting that NF-κB aggravates the inflammatory response via the upregulation of CXCL16, TNF-α and ICAM-1 (42). Lehrke *et al* (43) demonstrated that CXCL16 expression is significantly upregulated following stimulation with LPS and downregulated following treatment with NF-κB-targeting anti-inflammatory drugs *in vivo*. In addition, CXCL16 expression is significantly downregulated following treatment with the NF-κB inhibitor, SN50. However, Lehrke *et al* (43) also revealed that following activation of NF-κB, CXCL16 expression is significantly upregulated by overexpression of activated IκB kinase *in vitro*. In addition, Izquierdo *et al* (44) demonstrated that TNF-like weak inducer of apoptosis upregulated CXCL16 expression *in vivo* and *in vitro* via activation of the NF-κB transcription factor; however, expression of CXCL16 was revealed to be downregulated via inhibition of NF-κB activation, which demonstrated that the expression of CXCL16 is NF-κB-dependent. The present study demonstrated that the expression levels of NF-κB and CXCL16 were significantly increased in atherosclerotic ApoE-/- mice compared with control ApoE-/- mice, which also suggested that CXCL16 may represent a positive feedback mechanism to NF-κB in atherosclerotic diseases *in vivo*. Thus,

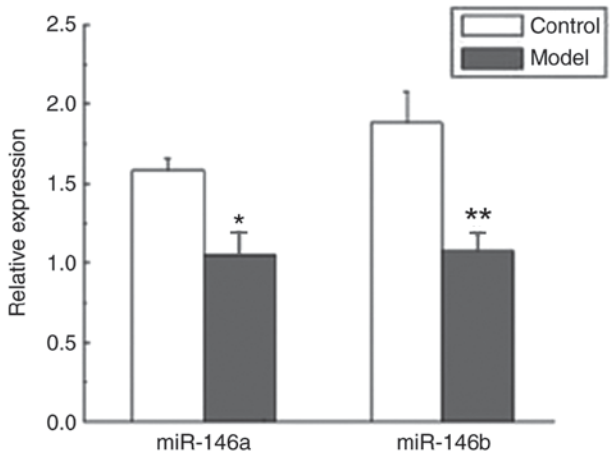

Figure 3. Expression levels of miR-146a and miR-146b. Data are presented as the mean ± standard deviation (n=6). *P<0.05 and **P<0.01 vs. control group. miR, microRNA.

it was demonstrated that the role of CXCL16 in atherosclerotic diseases may be closely associated with NF-κB.

The present study also revealed that the expression levels of TRL4, IRAK1 and TRAF6 were significantly increased in atherosclerotic ApoE-/- mice compared with control ApoE-/- mice. IRAK1 and TRAF6 are important adapter molecules in the TLR4/NF-κB pathway that can be activated by TLR4, which subsequently activates the NF-κB pathway (45). TLR4 can activate NF-κB pathways and increase the expression levels of IL-6 and IL-8 (46). Overexpression of TRAF6 and/or IRAK1 in mature dendritic cells by lentivirus transduction has been revealed to increase the expression level of NF-κB (27). The present study demonstrated that the expression levels of upstream and downstream factors of the NF-κB signaling pathway, such as TLR4, IRAK1, TRAF6, CXCL16, TNF-α and IL-1β, were significantly increased in atherosclerotic ApoE-/- mice compared with control ApoE-/- mice. These results demonstrated that the important role of CXCL16 in atherosclerosis may be via the TRL4/NF-κB/CXCL16 pathway.

In recent years, numerous studies have suggested that the expression levels of miRNAs are associated with human diseases, particularly diseases involving an inflammatory response. For example, decreased expression of let-7 in lung cancer tissues may promote the expression of Ras, and therefore Ras may be involved in the mechanism underlying the association between let-7 and lung cancer (47). Furthermore, expression of miR-181 has been revealed to be decreased in the aortic intima of ApoE-/- mice administered with high fat diets, which may promote the formation of atherosclerosis (48). Downregulation of miR-149 in osteoarthritism chondrocytes has been previously revealed be correlated with increased expression of pro-inflammatory cytokines (49). According to numerous previous studies, miRNA networks have important roles in the inhibition of inflammatory responses (50-52). For example, decreased expression levels of miR-10a and miR-181b have been revealed to be associated with inhibition of the development of atherosclerosis (51,52).

Numerous studies have demonstrated that miR-146a and miR-146b serve critical roles in the inflammatory response (53-55). Cheng *et al* (54) revealed that miR-146a can inhibit the NF-κB pathway, which may suppress the inflammatory response *in vitro*. Cheng *et al* (54) also demonstrated that miR-146b may regulate endothelial activation that represses the inflammatory response via inhibition of the NF-κB pathway activation, which suggested that increased expression levels of miR-146a or miR-146b in the vasculature may represent an effective treatment for the inhibition of the inflammatory response. Park *et al* (27) demonstrated that miR-146a and miR-146b regulate cell apoptosis and cytokine production via TRAF6 and IRAK1, and thus function as negative feedback mechanisms. Overexpression of miR-146a has been revealed to suppress NF-κB activity via decreasing IRAK1 and TRAF6 expression levels in the myocardium and attenuating the production of inflammatory cytokines, such as TNF-α, IL-1β and ICAM-1 (56). Curtale *et al* (23) demonstrated that miR-146b represents an IL-10-dependent regulator of the TLR4 signaling pathway that directly targets multiple elements, including IRAK1 and TRAF6. Furthermore, Curtale *et al* (23) revealed that increased expression of miR-146b markedly suppresses levels of proinflammatory cytokines, including CXCL10, TNF-α, IL-6 and IL-8 (23). The present study demonstrated that the expression levels of miR-146a and miR-146b were significantly downregulated in atherosclerotic ApoE-/- mice compared with control ApoE-/- mice; however, NF-κB and its downstream products were demonstrated to be significantly upregulated in atherosclerotic ApoE-/- mice compared with control ApoE-/- mice. This suggested that the downregulation of miR-146a and miR-146b expression levels may reduce the inhibitory effect on CXCL16 via the TLR4/NF-κB signaling pathway.

In conclusion, the present study revealed that the expression levels of CXCL16 and proteins associated with the TLR4/NF-κB signaling pathway were significantly upregulated, and the expression levels of miR-146a and miR146b were significantly downregulated, in atherosclerotic ApoE-/- mice compared with control ApoE-/- mice *in vivo*. The results of the present study suggested that CXCL16 may be associated with the TRL4/NF-κB/CXCL16 signaling pathway, and that miR-146a and miR-146b may negatively regulate CXCL16 via TLR4/NF-κB/CXCL16 signaling pathway in atherosclerosis *in vivo*. Therefore, enhanced expression of miR-146a and miR146b may represent an effective approach for the reduction or prevention of the inflammatory response in patients with atherosclerosis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

AM and XZ conceived, designed and performed the experiments, analyzed the data and drafted the manuscript. SY and TW performed the experiments and analyzed and interpreted the data. YW and XX also performed the experiments. SL provided experimental technical support. XP provided the concept and design of the study, together with critical revision of the manuscript for content, and obtained funding performed the administration.

Ethics approval and consent to participate

The study protocol was approved by the Animal Ethics Committee of Qingdao University prior to experimentation.

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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