Systemic delivery of rAAV9-IkB α stabilized atherosclerotic plaques and attenuated intra-plaque inflammation by inhibiting nuclear factor- κ B activation in apolipoprotein E-deficient mice

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Research article

Keywords: Atherosclerosis, AAV9-IkB α , Inflammation, NF- κ B, Plaque Stability

Posted Date: July 28th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-748433/v1>

Abstract

Background: The activated nuclear factor (NF)- κ B pathway in atherosclerotic plaques promotes the progression of atherosclerosis. Targeting of plaque NF- κ B may provide a novel strategy for limiting chronic inflammation. This study was conducted to examine the effect of NF- κ B inhibition by using recombinant adeno-associated virus 9 (AAV9) to deliver I κ B α (AAV9-I κ B α) which can be overexpressed to influence atherosclerosis.

Methods and Results: Systemic delivery of AAV9-I κ B α resulted in ~2-fold overexpression of I κ B α protein in the atherosclerotic plaques of apolipoprotein E^{-/-} mice fed a high-fat diet. AAV9-I κ B α delivery did not affect the body weight or lipid deposition and plaque size in the mice. During plaque formation, overexpression of I κ B α suppressed intra-plaque macrophage infiltration, inhibited the expression of pro-inflammatory genes including interleukin-6, monocyte chemoattractant protein-1, tumor necrosis factor- α and matrix metalloproteinase, enhanced the contents of collagen and vascular smooth muscle cells, and decreased the plaque vulnerability index by inhibiting the phosphorylation of NF- κ B subunit p65 and its nuclear translocation.

Conclusions: Systemic delivery of AAV9-I κ B α promotes plaque stability by inhibiting NF- κ B activation and suppressing intra-plaque inflammation, suggesting that inhibition of the NF- κ B pathway in atherosclerotic plaques is a promising approach for treating atherosclerosis.

Background

Atherosclerosis is the main cause of coronary heart diseases and constitutes a major healthcare burden worldwide. The inflammatory response is recognized as a key factor in the progression of atherosclerosis[1, 2]. Nuclear factor- κ B (NF- κ B) signaling has been implicated in various inflammatory pathologies including vascular diseases. The transcriptional activity of NF- κ B can be induced by numerous atherogenic stimuli such as enhanced oxidized low-density lipoprotein, inflammatory cytokines, integrin/matrix signaling activation, bacterial and viral infections, hemodynamic stress, and metabolic disorders[3–10].

In the canonical NF- κ B signaling pathway, NF- κ B heterodimers remain in an inactive form in the cytoplasm while adhered to an inhibitor such as I κ B α . Upon activation by pathological stimuli, the I κ B kinase (IKK) complex rapidly phosphorylates I κ B α , which interacts with the proteasome and leads to I κ B α degradation[11, 12]. When NF- κ B heterodimers are released from I κ B α , importin proteins (also known as karyopherins) guide translocation of NF- κ B to the nucleus where it controls the expression of a large number of genes by combining to numerous κ B elements. In the vascular endothelium and atherosclerotic plaques, NF- κ B activation induces the expression of proinflammatory genes encoding cytokines, adhesion molecules, and chemoattractant proteins which jointly play important roles in atherosclerosis progression[8, 13, 14]. Previous studies demonstrated that NF- κ B-specific inhibition in endothelial cells (ECs) in various genetic-modified mouse models (i.e. IKK γ EC knockout, IKK γ EC–

inducible knockout, and dominant-negative I κ B α EC transgenic) alleviated atherosclerosis[15]. Moreover, genetically inhibiting several NF- κ B-targeted genes including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1, E- and P-selectins, interleukin-1 beta (IL-1 β), and tumor necrosis factor- α (TNF- α) decreases various aspects of atherosclerotic lesion formation[16–20]. Thus, targeting NF- κ B activity shows potential for the development of novel anti-inflammatory therapies for acute and chronic inflammatory diseases.

Although several studies have evaluated the role of NF- κ B in atheromatous plaque formation, the effects of I κ B α activation in NF- κ B inhibition and atherosclerotic plaques remain unclear. Our previous study showed that recombinant adeno-associated virus 9 (rAAV9) vectors with a human cytomegalovirus (CMV) promoter produced using the recombinant baculovirus system effectively achieved long-term expression in atherosclerotic plaques[21]. In this study, we used the same tool to investigate whether overexpression of I κ B α in atherosclerotic plaques effectively suppresses inflammatory responses and therefore stabilizes plaques in apolipoprotein E-deficient (ApoE $^{-/-}$) mice, a well-established mouse model of atherosclerosis.

Methods

Ethics

Adult male ApoE $^{-/-}$ mice (aged 8 weeks) were used in this study. All experimental and animal care procedures were performed according to the Animal Management Rule of the Chinese Ministry of Health (edition 55, 2001), and were approved by the Animal Ethics Committee of Xinjiang Medical University.

Vector construction

The rAAV9 vector was purchased from Virovek (Hayward, CA, USA) and produced using the recombinant baculovirus-based system in SF9 cells, as previously described[22–24]. rAAV9 vectors were composed of single-stranded DNA containing the enhanced green fluorescent protein (GFP) gene (rAAV9-CMV-GFP: AAV9-GFP) or I κ B α gene (rAAV9-CMV-I κ B α : AAV9-I κ B α). Vector titers were determined by quantitative polymerase chain reaction (PCR) as previously described with primers corresponding to the CMV enhancer region[21, 25, 26].

Animals and grouping

ApoE $^{-/-}$ mice were bred and housed in a specific pathogen-free barrier facility at 22–25°C. Animals were housed in standard cages and kept on a 12-h light/12-h dark cycle with food and water freely available. All animals were first administered a high-fat diet (0.25% cholesterol and 21% fat) for 12 weeks followed by three different treatments for another 5 weeks: (1) control group (n = 25): mice were administered saline (100 μ L saline once) via the tail vein; (2) AAV9-GFP group (n = 25): mice were injected with AAV9-GFP (5.0×10^{11} vg/mouse/100 μ L) via the tail vein, and (3) AAV9-I κ B α group (n = 25): mice were injected with AAV9-I κ B α (5.0×10^{11} vg/mouse/100 μ L) according to our previous study[21]. Mice were co-injected

with AAV9-GFP and AVV9-IkB α via the tail vein at the doses described above. All mice were fed the high-fat diet throughout the experimental period (17 weeks).

Tissue preparation and quantification of atheroma (plaque) size

At 5 weeks after injection, the mice were starved for 12 h and then anesthetized with mixture of ketamine (100 mg/kg), xylazine (20 mg/kg), and atropine (1.2 mg/kg) via intraperitoneal injection. Blood samples were collected by cardiac puncture and cardiac palpation was used to confirm the death of mice. The chest was opened, the mice were perfused with phosphate-buffered saline through the left ventricle, and then 4% formaldehyde (pH 7.2) was perfused for 15–20 min. The aorta from the aortic arch to the iliac bifurcation and the left common carotid artery with bifurcation were isolated, immersed in 4% formaldehyde overnight (4°C), and then embedded in optical coherence tomography compound (OCT, Sakura Finetek, Inc., Torrance, CA, USA) and stored at -80°C until use[21]. Each vessel covering the entire length of the carotid artery was subjected to histological analysis.

Biochemical analysis

The serum was separated by centrifugation at 600 $\times g$ for 10 min at 4°C. The serum levels of total cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein cholesterol were measured using an automatic biochemical analyzer (HITACHI-7600; Hitachi, Ltd., Tokyo, Japan) in the hospital.

Histological analysis

Conventional staining: Cross-sections (6- μ m-thick) were cut at intervals of 50 μ m along the carotid artery specimens. The site containing the largest plaque was selected for analysis. The aortic arches were prepared and analyzed as previously described. Sections were stained with hematoxylin and eosin for general observation and with Sirius red staining for collagen quantification. Lipid deposition was identified by Oil-red O staining. Positively stained areas in the inner-surface area of the aorta were measured and expressed as a percentage of the total aortic area.

Immunohistochemistry: Corresponding sections on separate slides were stained with the following antibodies: monocyte/macrophage monoclonal antibody (MOMA-2, 1:50, Serotec, Oxford, UK), α -smooth muscle actin monoclonal antibody (1:50), IL-6 polyclonal antibody (1:100), TNF- α monoclonal antibody (1:100), anti-monocyte chemoattractant protein-1 (MCP-1) antibody (1:200), and matrix metalloproteinase (MMP-2) monoclonal antibody (1:100, all from Abcam, Cambridge, UK). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies, the sections were incubated with 3',3'-diaminobenzidine and counterstained with hematoxylin. Sections reacted with non-immune IgG and secondary antibodies were used as negative controls. Positive staining areas of macrophages, smooth muscle cells (SMCs), lipids, collagen, IL-6, MCP-1, TNF- α , or MMP-2 were quantified using a microscope (Leica 3000, Wetzlar, Germany) with Image-Pro Plus software (version 5.0, Media Cybernetics, Inc., Rockville, MD, USA). The percentage of the positive staining area to the plaque

area was calculated. The vulnerable index was calculated using the following formula: relative positive staining area of (macrophages% + lipid%)/ relative positive staining area of (α -SMCs% + collagen%)[27].

Immunofluorescent staining: Immunofluorescent staining was performed to identify NF- κ B subunit p65 localization in the carotid artery specimens. Frozen sections were incubated with 3% H₂O₂ for 10 min, followed by blocking with sealing fluid for 30 min. The sections were then incubated overnight at 4°C with an NF- κ B p65 antibody (1:50, rabbit polyclonal, Thermo Fisher Scientific), followed by incubation with goat anti-rabbit IgG, dylight TM 594 (1:100, goat anti-rabbit IgG, Thermo Fisher Scientific). The sections were counter-stained with DAPI to identify the nuclei before mounting. The sections were used for sequential double immunofluorescence staining and then counter-stained for observation by confocal microscopy.

Quantitative real-time PCR

Real-time quantitative PCR was performed to assess the expression of various genes. Total RNAs were extracted from the total aorta of mice using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Next, 1 μ g DNase I-treated (Thermo Fisher Scientific) total RNA was reverse-transcribed using the Primer Script Strand cDNA Synthesis Kit (Promega, Madison, WI, USA). Reverse transcription and real-time fluorescent quantization were performed according to the manufacturer's instructions. The PCR device (Bio-Rad MyCycler), electrophoresis apparatus trophoresis (DYY-6D, Liuyi Brand, Japan), and high-speed freezing centrifuge (HC-3018R) were used. Samples were evaluated in triplicate. The housekeeping GAPDH gene was used for internal normalization. The mean SQ values of the target gene primers were compared to those of GAPDH-specific primers using the double standard curve method. The data are presented as fold-changes of transcripts for the target gene normalized to GAPDH compared to in control mice. Primers were designed to amplify mouse IkB α , IL-6, TNF- α , MCP-1, MMP-2, and GAPDH. All primers (Table 1) were obtained from Shanghai Sangong Company (Shanghai, China). The reaction was performed under the following conditions: 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. A DNA purification kit was used (Tiangen Biotech Co., Ltd., Beijing, China), cDNA was amplified using a Quanti Fast SYBR® Green Real-time PCR Master Mix (Qiagen, Hilden, Germany) in 96-well optical reaction plates on an fluorescent quantization PCR device (CFX96, Bio-Rad) according to the manufacturer's protocol. The cycling parameters were as follows: 95°C for 5 min and then 40 cycles of 95°C for 10 s and 60°C for 30 s, followed by melting curve analysis. Quantitative values were obtained from the threshold cycle value (Ct) and the data were analyzed by the $2^{-\Delta\Delta C_T}$ method. The housekeeping gene GAPDH was amplified and quantified as an internal control.

Western blot analysis

Protein extracted from fresh aorta plaques were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk, and then washed with phosphate-buffered saline containing 0.1% Tween 20 and incubated with an appropriate primary antibody at 4°C overnight. The blots were probed with antibodies against GAPDH

(1:3,000), I κ B α (1:200), P-P65 (1:100), P65 (1:200), and P50 (1:200), which were obtained from Cell Signaling Technology (Danvers, MA, USA). After overnight incubation, the blots were washed with Tris-buffered saline-Tween-20 and incubated with secondary antibodies at room temperature for 2 h, followed by three washes for 10 min each membranes and detection using the ECL Western blot detection system. Quantity One software (Bio-Rad) was used for analysis; the integral optical density value was calculated as the target protein divided by the internal reference GAPDH protein. The relative values of protein bands in each group were expressed as the mean $\pm$ standard deviation.

Statistics analysis

Statistical analysis was performed with SPSS 22.0 software (SPSS, Inc., Chicago, IL, USA). After the numeric variable data were tested for normality and homogeneity of variance, all parameters determined in this study were presented as the mean $\pm$ standard deviation. Differences among groups were compared by general linear model-univariate or one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Systemic delivery of AAV9-I κ B α resulted in overexpression of I κ B α in atherosclerotic plaques

First, we examined I κ B α expression in aortic plaques in three different groups (control, AAV9-GFP, and AAV9-I κ B α groups) at 5 weeks after tail vein injection. There were no differences in I κ B α protein or gene expression between the control and AAV9-GFP groups. In the AAV9-I κ B α group, the protein and mRNA levels of I κ B α were increased by 1- and 4.7-fold compared to control values, respectively (Figure 1, both $P < 0.05$). These results indicate that single injection of the AAV9 delivery system can achieve stable and effective overexpression of a targeted gene at a specific site.

Systemic delivery of I κ B α did not influence animal body weight or lipid profile

Five weeks after transfection of I κ B α , body weight and circulating lipid levels were comparable among the three groups (Table 2), suggesting that I κ B α gene transfection did not significantly affect body weight and the circulating lipid profile in atherosclerotic mice.

Overexpression of I κ B α stabilized atherosclerotic plaques without influencing plaque size

Histological and immunohistochemical studies revealed the accumulation of macrophages as identified by MOMA-2, lipids as detected by oil-red O staining, collagen as detected by Sirius red staining, and SMCs detected by evaluating α -smooth muscle actin in atherosclerotic plaques of the carotid artery. In macrophages, although lipids, collagen, and SMCs showed comparable contents between the control and AAV9-GFP groups, AAV9-I κ B α gene transfection significantly reduced the accumulation of macrophages and increased collagen deposition and SMCs in the plaques (Figure 2, all $P < 0.05$). These changes reduced by plaque vulnerability index by more than 50% in the AAV9-I κ B α group versus in the control and AAV9-GFP groups (Figure 2). Unexpectedly, AAV9-I κ B α gene transfection did not influence the size of

atherosclerotic plaques in the whole aorta, as evidenced by similar lesion areas stained with oil-red O (Figure 3); comparable lipid accumulation was also observed in the plaques (Figure 2).

Overexpression of I κ B α suppressed expression of inflammatory mediators in the plaques

Considering the crucial role of inflammation in the pathogenesis of atherosclerosis, we further examined whether I κ B α gene delivery regulates vascular inflammatory responses in the atherosclerotic lesions. There was no difference in the expression of IL-6, TNF- α , MCP-1, and MMP-2 between the control and AAV9-GFP groups according to immunohistochemistry analysis and real-time PCR to examine mRNA levels (Figure 4). However, AAV9-I κ B α gene delivery significantly decreased the positively stained areas of IL-6, TNF- α , MCP-1, and MMP-2 (approximately 50% reduction) compared to control levels, showing similar decreases in the mRNA levels of these genes (Figure 4). These results suggest that I κ B α overexpression suppressed inflammatory responses in atherosclerotic lesions.

Overexpression of I κ B α suppressed NF- κ B pathway in atherosclerotic plaques

We next examined the effect of I κ B α gene delivery on NF- κ B inhibition. As expected, corresponding to increased expression of I κ B α in aortic plaques (Figure 1), phosphorylation of the NF- κ B subunit p65 was decreased in the AAV9-I κ B α group compared to in the control and AAV9-GFP groups (Figure 5A and B, $P < 0.05$). Our immunofluorescence staining results showed that the density of p65 overlapping with the nuclei (stained by DAPI) was decreased by 30% compared to the control level (Figure 5C and D). However, there were no differences in these parameters between the control and AAV9-GFP groups. There was no significant difference in p65 and p50 protein expression, whereas AAV9-I κ B α effectively inhibited the expression of NF- κ B nuclear protein p-p65 (Figure 5A–D).

Discussion

We previously showed that recombinant adeno-associated virus 9 (rAAV9) vectors with a human cytomegalovirus promoter produced with the recombinant baculovirus system effectively achieved long-term expression in atherosclerotic plaques. Our study is the first study, we used this system to investigate overexpression of I κ B α in atherosclerotic plaques effectively suppresses inflammatory responses and stabilizes plaques in apolipoprotein E-deficient (ApoE $^{-/-}$) mice. In this study, we investigated the effectiveness of systemic delivery of AAV9-I κ B α in inhibiting NF- κ B activation and influencing atherosclerotic plaque stability and related inflammation. We believe that our study makes a significant contribution to the literature because we found that systemic delivery of I κ B α by AAV9 resulted in stable and effectively enhanced expression of I κ B α in atherosclerotic lesion, resulting in stabilization of plaques and attenuating the expression of inflammatory mediators. Thus, inhibiting NF- κ B activation in atherosclerotic plaques is a potential therapeutic approach for treating atherosclerosis.

AAV9-I κ B α also had no effect on atherosclerotic lesion size or the lipid component in plaques (Fig. 3), which is a limitation of the current study. A higher dose may lead to higher expression levels of the targeted gene, which may yield greater protective effects and explain why the lesion size was not

reduced. The enhanced expression of I κ B α in the present study resulted in plaque stabilization, as evidenced by decreased macrophage accumulation and increased collagen deposition and SMC numbers. The protective role of I κ B α in atherosclerosis has been documented in numerous studies. For example, myeloid I κ B α deficiency promoted atherogenesis by enhancing leukocyte recruitment to developing plaques[28]. Overexpression of DN I κ B α resulted in more dramatic, nearly complete inhibition of atherosclerotic plaque growth and vulnerability in ApoE^{-/-} mice, which may be because of incomplete ablation of NEMO in the endothelial lining of the arterial wall[15]. However, in contrast to the findings showing that I κ B α overexpression inhibited early aortic lesion formation, our study revealed that systemic transgenic overexpression of I κ B α did not affect the size of advanced carotid plaques in mice. This may be because relatively short-term manipulation of I κ B α expression in an already established plaque is not adequate for inducing atherosclerotic regression. Further studies are needed to address this important issue.

The NF- κ B signaling pathway plays a critical role in regulating the expression of multiple genes involved in the progression of atherosclerosis including inflammatory-related genes/mediators. Previous studies indicated that inhibition of NF- κ B in specific cell types has discrepant effects. Although EC-specific inhibition of NF- κ B resulted in reduced lesion formation in ApoE^{-/-} mice[15, 29, 30], the effect of altering NF- κ B activation in myeloid cells is more complicated. For example, LDL receptor-deficient mice transplanted with IKK2/IKK β -deficient macrophages showed increased atherosclerosis lesions, which was associated with increased numbers of apoptotic cells in the plaque[31]. In contrast, myeloid-specific IKK β deficiency reduced atherosclerosis in LDL receptor-deficient mice[32]. Another study showed that myeloid I κ B α deficiency increased atherogenesis by promoting leukocyte aggregation to advanced plaques[28]. Moreover, bone marrow deficiency of NF- κ B1 led to reduced atherosclerotic plaque sizes and macrophage foam cells but resulted in enhanced lesion inflammation[33]. These data illustrate that cell-type-specific inhibition upstream of the NF- κ B pathway may have diverse effects on the plaque composition and atherosclerotic lesion formation.

Our results suggest that systemic delivery of I κ B α mediated the inhibition of NF- κ B activation by attenuating the phosphorylation and nuclear translocation of NF- κ B p65 subunit. The atheroprotective role of NF- κ B inhibition has been reported in different mouse models. NEMO ablation interferes with IKK activation or transgenic expression of degradation-resistant DN I κ B α blocks nuclear translocation of NF- κ B[15]. Pharmacological inhibition of NF- κ B in human vascular SMCs maintained cytosolic levels of I κ B α which paralleled the decreased p65 nuclear translocation[34]. These results agree with our current findings showing that enhanced I κ B α expression in the lesion inhibited NF- κ B activation, thereby alleviating atherosclerosis. Moreover, our recent study revealed that downregulation of the NF- κ B p50 subunit led to endothelial dysfunction and enhanced inflammatory and apoptosis, which was associated with the risk and severity of coronary heart disease[35]. Taken together, these results demonstrate a critical role for NF- κ B in the development of atherosclerosis and indicate that inhibition of NF- κ B by gene therapy is a promising approach for limiting atherosclerosis. Two distinct pools of I κ B α are known to exist in cells; the larger I κ B α pool is associated with NF- κ B and the minor pool remains as 'free'

protein[36]. I κ B α is an unstable protein whose rapid degradation is slowed upon binding to NF- κ B; bound I κ B α then requires IKK phosphorylation and ubiquitination for slow basal degradation. Altering free I κ B α degradation dampens NF- κ B activation[37]. This steady-state level of total I κ B α may partially explain how overexpression of I κ B α inhibits NF- κ B activation.

The current study has several limitations. I κ B α mediates NF- κ B to regulate inflammatory factors, whether there is a feedback regulation between inflammatory factors and NF- κ B, and the effect of overexpression of I κ B α on the corresponding tissues other than blood vessels need further investigation. There are a variety of cells in atherosclerotic plaques in this study, and it is still necessary to further clarify the types of cells targeted by the virus and the specific functions of the cells affected by the intervention. Macrophages play a key role in the occurrence and development of AS. The study on the effect of I κ B α on macrophages and the effect on liver glucose and lipid metabolism will also be an important target of anti-inflammatory and anti-AS, which is worth further exploration.

Conclusions

In summary, systemic delivery of I κ B α by AAV9 achieved stably and effectively enhanced expression of I κ B α in atherosclerotic lesion, resulting in stabilization of plaques and attenuating the expression of inflammatory mediators. These results suggest that inhibition of NF- κ B activation in atherosclerotic plaques is a potential therapeutic approach for treating atherosclerosis.

Declarations

Ethics approval and consent to participate

All experimental and animal care procedures were performed according to the Animal Management Rule of the Chinese Ministry of Health (edition 55, 2001), and were approved by the Animal Ethics Committee of Xinjiang Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by a project grant of Natural Science Foundation of China (ID 82070368). There are no relationships with the company relating to employment, consultancy, patents, products in development or marketed products. The funding body had influence on designing research, data collection, data analyzing, and writing the manuscript.

Authors' contributions

Study design: YNY, XMG, XML. Performed experiments: QJC, JX, FL. Data analysis: XMW, YTM, BDC, LZ, HZ. Manuscript drafting: QJC. Quality control of the study and critical revision: XMG. All authors have read and approved the manuscript.

Acknowledgements

The authors wish to thank the Department of Cardiology of Qilu Hospital of Shandong University for help with the experiments.

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Abbreviations

AS: Atherosclerosis; ApoE: Apolipoprotein E; HDL-C: High density lipoprotein cholesterol; Ldlr: Low density lipoprotein receptor; IL-1: Interleukin-1; IL-6: Interleukin-6; IκB: Inhibitor kappaB; LDL-C: Low-density lipoprotein cholesterol; MMP-2: Matrix metalloproteinase-2; NF-κB: Nuclear factor kappa B; rAAV9: Recombinant adeno-associated virus serotype 9; TC :Total cholesterol; TG: Triglyceride; TNF-α: Tumor necrosis factor-alpha.

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Tables

Table 1. Sequences of examined primers.

Gene	Forward	Reverse
GAPDH	5'-GGTGAAGGTCGGTGTGAACG-3'	5'-CTCGCTCCTGGAAGATGGTG-3'
IkBα	5'-AGCATCTCCACTCCGTCCT-3'	5'-AGCACCCAAAGTCACCAAGT-3'
TNF-α	5'-TCTCATGCACCACCATCAAG-3'	5'-GAGGCAACCTGACCACTCTC-3'
IL-6	5'-CGGAGAGGAGACTTCACAGAG-3'	5'-CATTTCCACGATTTCCCAGA-3'
MCP-1	5'-CCACTCACCTGCTGCTACTC-3'	5'-ACAGCTTCTTTGGGACACCT-3'
MMP-2	5'-GCCAAGGTGGAAATCAGAGA-3'	5'-GTTGAAGGAAACGAGCGAAG-3'

Table 2. Effects of systemic gene delivery on body weight and lipid profile.

Group	BW	TC	TG	HDL-C	LDL-C
	g	mmol/L	mmol/L	mmol/L	mmol/L
Control	30.5 ± 3.1	20.32 ± 2.41	1.54 ± 0.10	1.26 ± 0.15	6.73 ± 0.36
AAV9-eGFP	29.8 ± 2.2	19.86 ± 1.97	1.49 ± 0.09	1.12 ± 0.10	6.50 ± 0.67
AAV9-IκBα	30.9 ± 2.8	20.64 ± 1.46	1.52 ± 0.14	1.29 ± 0.18	6.47 ± 0.22

Data are expressed as the mean ± SE. n = 25 per group. AAV9-GFP; AAV9-IκBα; BW, body weight; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. There was no significant difference in any parameters among the 3 groups.

Figures

Figure 1

IκBα overexpression in the plaque. A, Representative western blot bands of IκBα in control, AAV9-GFP, and AAV9-IκBα groups. B, Quantitative data of IκBα protein expression in the 3 groups. n = 5 per group. C, mRNA levels of IκBα expression determined by real-time PCR in the 3 groups. n = 7 per group. *P<0.01 vs control or AAV9-GFP group.

Figure 2

Effects of IκBα overexpression on plaque composition and vulnerability index in 3 groups of mice. A, Staining of carotid plaques, macrophages, lipids, collagen, and SMCs (×400); Arrowheads indicate positively stained areas. B, Quantitative analysis of carotid plaque composition in A. C, Vulnerability index in 3 groups. Data are shown are the mean ± SEM (n = 7 mice per group). *P < 0.01 vs control group or AAV9-GFP group.

Figure 3

Local lesion area and whole aorta lesion lipid area in 3 groups of mice. A, HE staining of brachiocephalic artery ($\times 200$). B, Whole surface of aorta stained by Oil-red O. C, HE staining measurements in 3 treatment groups. D, Oil-red O staining measurements (%) in 3 treatment groups. Data shown are the mean $\pm$ SEM ($n = 5-7$ mice per group). There was no significant difference between the 3 groups in HE and Oil-red O staining, $P > 0.05$.

Figure 4

Effects of IκBα overexpression on plaque inflammatory cytokine expression in 3 groups of mice. A, Staining for carotid plaques, IL-6, MCP-1, TNF-α, and MMP-2 in 3 treatment groups (×200); arrowheads indicate positive staining areas. B, Quantitative analysis of the carotid plaque composition in A. C, mRNA expression levels of IL-6, TNF-α, MCP-1, and MMP-2 in 3 treatment groups. Data shown are the mean ± SEM (n = 5–7 mice per group). *P < 0.01 vs control group or AAV9-GFP group.

Figure 5

Aorta and plaque protein expression levels of NF-κB signaling pathway in 3 groups of mice. A, Western blot analysis of IκBα, P-P65, P65 protein expression levels in 3 groups of mice. B, Quantitative analysis of the results in A. Data shown are the mean ± SEM (n = 5 mice per group). *P<0.05 vs control group or AAV9-GFP group. C, Frozen sections of brachiocephalic artery were stained with 4',6-diamidino-2-phenylindole (DAPI; blue) and anti-P65 (red). Arrows indicate differential p65 expression and p65 accumulation in nuclei of cells (Merger). D, Nuclear P65 expression in lesion was quantified. Data shown are the mean ± SEM (n = 5 mice per group). *P<0.05 vs control group or AAV9-GFP group.

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