

Lentiviral-mediated RNA interference of lipoprotein-associated phospholipase A₂ ameliorates inflammation and atherosclerosis in apolipoprotein E-deficient mice

HUI ZHANG, JINYING ZHANG, DELIANG SHEN, LI ZHANG, FEI HE, YUHUA DANG and LING LI

Department of Cardiology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, P.R. China

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Abstract. Lipoprotein associated phospholipase A₂ (Lp-PLA₂) overexpression is implicated in atherosclerosis. In the present study, we evaluated the effects of lentiviral-mediated RNA interference (RNAi) of Lp-PLA₂ on inflammation and atherosclerosis in apolipoprotein E-deficient mice. Apolipoprotein E-deficient mice were randomly allocated to control and experimental groups, and constrictive collars were used to induce plaque formation. Eight weeks after surgery, the lentiviral-mediated RNAi construct was used to silence expression of Lp-PLA₂. Control and experimental lentivirus was transfected directly into carotid plaques or administered systemically. Tissues were collected for analysis 7 weeks after transfection. Inflammatory gene expression in the plasma and atherosclerotic lesions was then determined at the mRNA and protein levels. We observed no differences in body weight and plasma lipid levels at the end of the investigation. However, the expression levels of Lp-PLA₂ and pro-inflammatory cytokines were significantly reduced in the RNAi groups, compared to the controls, whereas the plasma concentration of anti-inflammatory cytokines was markedly increased. Moreover, our results demonstrated a significant reduction in plaque area and lipid content, as well as a rise in collagen content following RNAi treatment. Importantly, when comparing the two methods of viral delivery, we found that transluminal local transfection exhibited enhanced improvement of plaque stability as compared to systemic administration. Inhibition of Lp-PLA₂ by lentiviral-mediated RNAi ameliorates inflammation and atherosclerosis in apolipoprotein E-deficient mice. In addition, transluminal local delivery of Lp-PLA₂ shRNA is superior to systemic administration for stabilizing atherosclerotic plaques.

Introduction

Despite major advances in the understanding and treatment of atherosclerosis, coronary heart disease continues to contribute to significant morbidity and mortality in the general population. It is increasingly recognized that inflammation plays an important role in the development of atherosclerosis (1-3). Lipoprotein-associated phospholipase A₂ (Lp-PLA₂), also termed platelet-activating factor acetylhydrolase (PAFAH), is one of the most studied circulating biomarkers of inflammation in the setting of atherosclerosis (2). This enzyme is predominantly associated with LDL in humans and HDL in mice, and increasing evidence suggests that it plays a pivotal role in the pathogenesis of atherosclerosis (4). For example, it is known to be an important predictor of atherothrombotic events and a direct participant in the formation of atherosclerosis (5). Biochemically, Lp-PLA₂ reacts with oxidized phospholipids to generate the pro-inflammatory by-products lysophosphatidylcholine (LPC) and oxidized non-esterified fatty acid (oxNEFA), both of which are implicated in the progression of atherosclerosis (5). Specifically, LPC is known to increase the expression of vascular adhesion molecules, to upregulate several cytokines and the CD40 ligand, and to stimulate macrophage proliferation, all of which play a critical role in atherosclerosis (6).

Multiple *in vitro* and *in vivo* studies have collectively suggested a causative role of Lp-PLA₂ in the development of atherosclerosis. Therefore, we hypothesized that the inhibition of its activity may have beneficial effects (5). The effect of RNA interference (RNAi) of Lp-PLA₂ on atherosclerosis in mouse models has not previously been studied, as such we used a lentiviral-mediated RNAi approach which has been proven to be efficacious in silencing target genes in dividing and nondividing cells (7,8). Traditional concepts of inflammation in atherosclerosis are regarded as an 'inside-out' responses, holding the central tenet that inflammatory responses are initiated at the intima. Therefore, we proceeded with transfection using the transluminal approach. As such, in the present study, we aimed to delineate the effect of lentiviral-mediated RNAi of Lp-PLA₂ on the progression of atherosclerosis and associated inflammation in apolipoprotein E-deficient mice, to further establish the role of Lp-PLA₂ in atherosclerosis.

Correspondence to: Professor Jinying Zhang, Department of Cardiology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, P.R. China
E-mail: jinyingzhang@yahoo.cn

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Materials and methods

Lentiviral vectors for Lp-PLA₂ RNAi. The target sequence (5'-GCAAGCTGGAATTCCTCTTG-3') within the murine Lp-PLA₂ mRNA was chosen as the target for RNAi. A scrambled shRNA sequence (5'-TTCTCCGAACGTGTCACGT-3') served as a negative control (NC). Vectors were constructed as previously described (9,10). The titers averaged 1×10^9 transduction units (TU)/ml.

Cell culture. The RAW 264.7 mouse macrophage cell line was routinely cultured in DMEM. When cells had grown to 90% confluence, Lp-PLA₂ interfering lentiviruses and lenti-scrambled-shRNA were then used to transfect RAW 264.7 cells at a multiplicity of infection (MOI) of 50. Previous studies demonstrated that unstimulated macrophage cells failed to produce detectable levels of Lp-PLA₂, while oxidized (ox)LDL upregulated the expression of Lp-PLA₂ in a concentration- and time-dependent manner (11). In our preliminary cell experiments, the expression of Lp-PLA₂ reached the platform stage after 60 μ g/ml of oxLDL stimulation. Therefore, we pretreated the cells with 60 μ g/ml oxLDL. Next we investigated the effects of RNAi on the expression of Lp-PLA₂, monocyte chemotactic protein-1 (MCP-1) and interleukin-6 (IL-6) by quantitative real-time PCR. Non-lentivirus and lentivirus-containing NC shRNA transfection served as controls.

Animals and experimental protocol. One hundred and four male apolipoprotein E-deficient mice received a high-fat diet (0.25% cholesterol and 15% cocoa butter) and underwent constrictive collar placement around the left common carotid artery after anesthesia with an intraperitoneal injection of pentobarbital sodium (30–50 mg/kg), using the method of von der Thüsen *et al* (12). In brief, the common carotid arteries were dissected and a constrictive silastic collar (0.30 mm) was placed on the left common carotid artery near its bifurcation by placement of 3 circumferential silk ties. The sham-operated group underwent cervical incision and closure without the placement of a constricting collar or instillation of a lentiviral suspension. Subsequently, the entry wound was closed and the animals were returned to their cage for recovery from anesthesia. A heating pad and a heating lamp were used to maintain body temperature. Eight weeks following surgery, the mice were randomly assigned to the following 5 groups: i) sham-operated group (n=18), without collar placement or lentiviral suspension instillation; ii) control group (n=18), the mice had their collars removed without virus infusion; iii) NC group (n=32), had their collars removed and received an infusion of 50 μ l (5×10^7 TU) NC viral suspension instilled into the left common carotid arteries via the external carotid under anaesthesia; the suspension was left *in situ* for 30 min and the skin incision was subsequently closed with silk sutures (12); iv) RNAi group 1 (RNAi1) (n=18), had their collars removed and received an intravenous (systemic) injection of 50 μ l RNAi viral suspension injected into the tail vein of mice and v) RNAi group 2 (RNAi2) (n=18), had their collars removed and received a local infusion of 50 μ l RNAi viral suspension instilled into the left common carotid arteries via the external carotid (13). To check the transfection efficiency of the lentivirus in atherosclerotic plaques, mice of the NC

group were sacrificed at a rate of 2 mice every week after transfection. Cryosections were observed with an Olympus microscope with fluorescent light to identify GFP expression. The remaining mice were all sacrificed at the end of week 15, and the left common carotid arteries were collected for histopathological analysis. The animal experimental protocol complied with the Animal Management Rules of the Chinese Ministry of Health (document no. 55, 2001) and was approved by the Ethics Committee of Zhengzhou University (Zhengzhou, China).

Histological analysis. The left common carotid artery was carefully excised, embedded in OCT compound, and underwent histological analysis for identification of the apex of the lesion, which displays the smallest lumen. Sections were stained with hematoxylin and eosin (H&E). Collagen and lipid deposition in plaques was identified by Masson's trichrome and Oil Red O (ORO) staining, respectively.

RNA extraction and RT-PCR. Total RNA was extracted with TRIzol reagent. Complementary DNA was synthesized using the reverse transcription kit (CoWin Bioscience Co., Ltd., Beijing, China). PCR products were synthesized using SYBR-Green RT-PCR Master Mix and were analyzed with a RT-PCR cyclor and detection system (ABI Prism 7300 Sequence Detection System; PE Applied Biosystems, Foster City, CA, USA). Quantitative values were obtained from the threshold cycle (Ct) value. The specific primer sequences were designed by Primer Premier 5 software. The specific primers used were as follows: 5'-ACAACCACGGCCTTCCCTACTT-3' and 5'-TTTCTCATTTCCACGATTTCCT-3' for IL-6; 5'-CTG GACAACATACTGCTAACCG-3' and 5'-TCAAATGCT CCTTGATTCTGG-3' for IL-10; 5'-CCAGAGATTTCAG ATGTGGAGTT-3' and 5'-TGGCAGAGTTGATAAAGA GGAG-3' for Lp-PLA₂; 5'-GCCTGACTCTGGTGATTT CTTG-3' and 5'-TGTTGATGTCTGCTTCTCCCTG-3' for MMP-8; 5'-GCTCAGCCAGATGCAGTTAACG-3' and 5'-TCTTGGGGTCAGCACAGACCTC-3' for MCP-1; 5'-TGT CTACTGAACCTCGGGGTGA-3' and 5'-TGTTTGTCTACG ACGTGGGCTA-3' for TNF- α ; and 5'-GCTATGCTCTCC CTCACGCCAT-3' and 5'-TCACGCACGATTTCCCTCTC AG-3' for β -actin. The results were analyzed by the $2^{-\Delta\Delta C_t}$ method, which reflects the difference in threshold for the target gene relative to that of β -actin.

Western blot analysis. Tissues were collected and lysed with protease inhibitor in 1X lysis buffer on ice for 10 to 15 min. Homogenates were centrifuged at 12,000 \times g for 20 min on ice, and the supernatants were collected and the protein content was quantified using the BCA method, and then SDS-PAGE electrophoresis was performed. Proteins were transferred to PVDF membranes. Membranes were then blocked with 5% non-fat milk and incubated overnight with primary antibodies against Lp-PLA₂, MMP-8 (Abcam, Cambridge, UK), TNF- α , IL-6 and β -actin (Zhongshan Biological Technology Co. Ltd, Beijing, China). After washing with TBS-T, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 60 min. Subsequently, the appropriate HRP-conjugated secondary antibodies were used, and the blots were probed and then exposed to X-ray film.

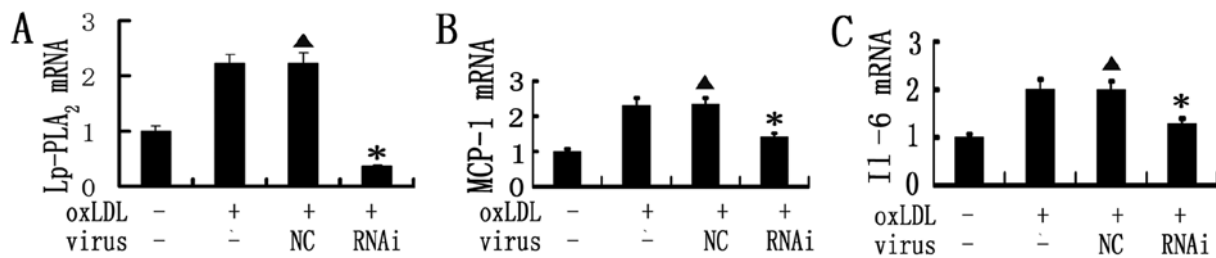


Figure 1. Real-time PCR quantification of Lp-PLA₂, MCP-1 and IL-6 mRNA expression in RAW 264.7 cells. After 60 μ g/ml of oxLDL pretreatment, the expression of Lp-PLA₂ increased sharply. Mouse RAW 264.7 cells were untransfected or transfected with NC LV or Lp-PLA₂ RNAi LV (MOI, 50) to determine their efficiency. (A) Compared with cells treated with oxLDL alone, cells treated with virus suspension demonstrated significant inhibition of Lp-PLA₂ expression. (B and C) RNAi inhibited the augmentation of MCP-1 and IL-6 induced by oxLDL. No significant differences were found between the control and NC group. Symbols - and + indicate the absence and presence of oxLDL or lentivirus. Data are the mean \pm SD from 3 independent experiments. * $P < 0.05$ vs. control groups; $^{\Delta}P > 0.05$ vs. control group.

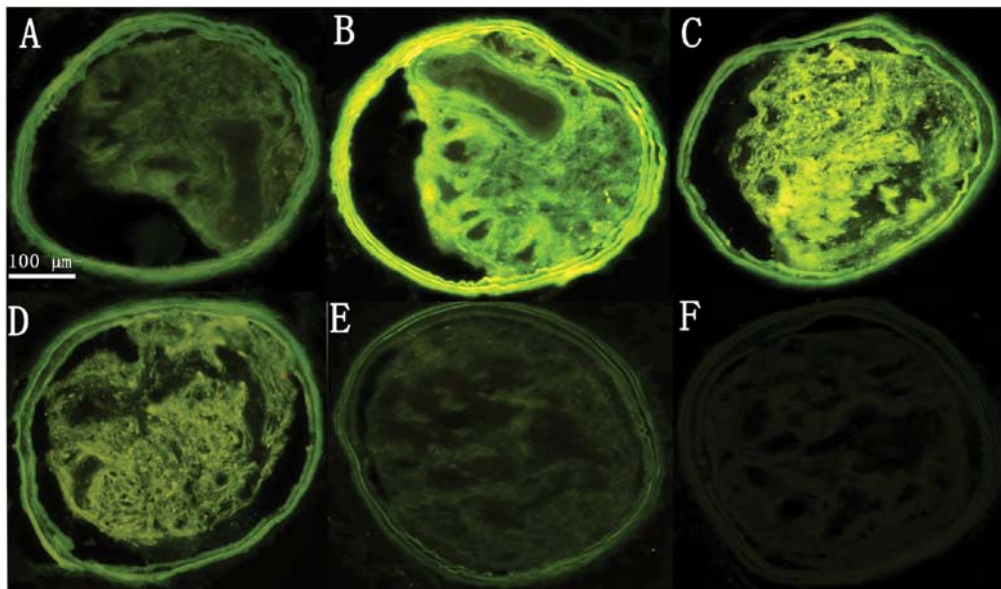


Figure 2. Efficiency of lentiviral transfection in carotid plaques. Fluorescence microscopy was used to identify GFP expression in the carotid plaques of the NC group. Fluorescent images of plaques were obtained at (A) 1 week, (B) 2 weeks, (C) 4 weeks, (D) 6 weeks and (E) 7 weeks after transfection, (F) Fluorescent image of lesion not treated with the lentivirus, indicating non-specific fluorescence. Magnification, $\times 200$.

Plasma lipid and biological analysis. Plasma concentration of Lp-PLA₂, IL-10, MMP-8, TNF- α , total cholesterol (TC), and triglyceride (TG) were measured using quantitative sandwich enzyme immunoassay (commercial ELISA kits) following the manufacturer's recommendation (CoWin Bioscience Co., Ltd.).

Statistical analysis. Data are presented as mean values \pm standard deviation (SD). Data were compared among groups using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) test for post-hoc comparisons. All statistical analyses were performed using SPSS version 16.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant result.

Results

Effects of RNAi on the expression of Lp-PLA₂ and pro-inflammatory cytokines in vitro. RAW 264.7 cells showed very low expression of Lp-PLA₂ before oxLDL stimulation.

After 60 μ g/ml of oxLDL pretreatment, the expression of Lp-PLA₂ increased sharply. Mouse RAW 264.7 cells were then transduced with MOI 50 of each vector to determine their efficiency. Our results demonstrated that Lp-PLA₂ RNAi led to an 83.8% ($P < 0.001$) decrease in Lp-PLA₂ mRNA expression in RAW 264.7 cells. As expected, no effect was observed following scrambled shRNA infection. In addition, our study demonstrated that Lp-PLA₂ RNAi inhibited the augmentation of MCP-1 and IL-6 induced by oxLDL (Fig. 1).

Safety and efficiency of RNAi in vivo. Following surgery and lentiviral infection, all mice were apparently healthy, and no animals died before the day of sacrifice. Previous studies have indicated that GFP expression provides an efficient and convenient way to detect the transfection efficiency of lentiviruses (7,13). Therefore, GFP fluorescence in carotid artery plaques was examined once a week after transfection (Fig. 2). Slight GFP fluorescence in the carotid plaques was displayed 1 week after transfection. The strongest GFP fluorescence was manifested 2 and 3 weeks after transfection. Modest GFP

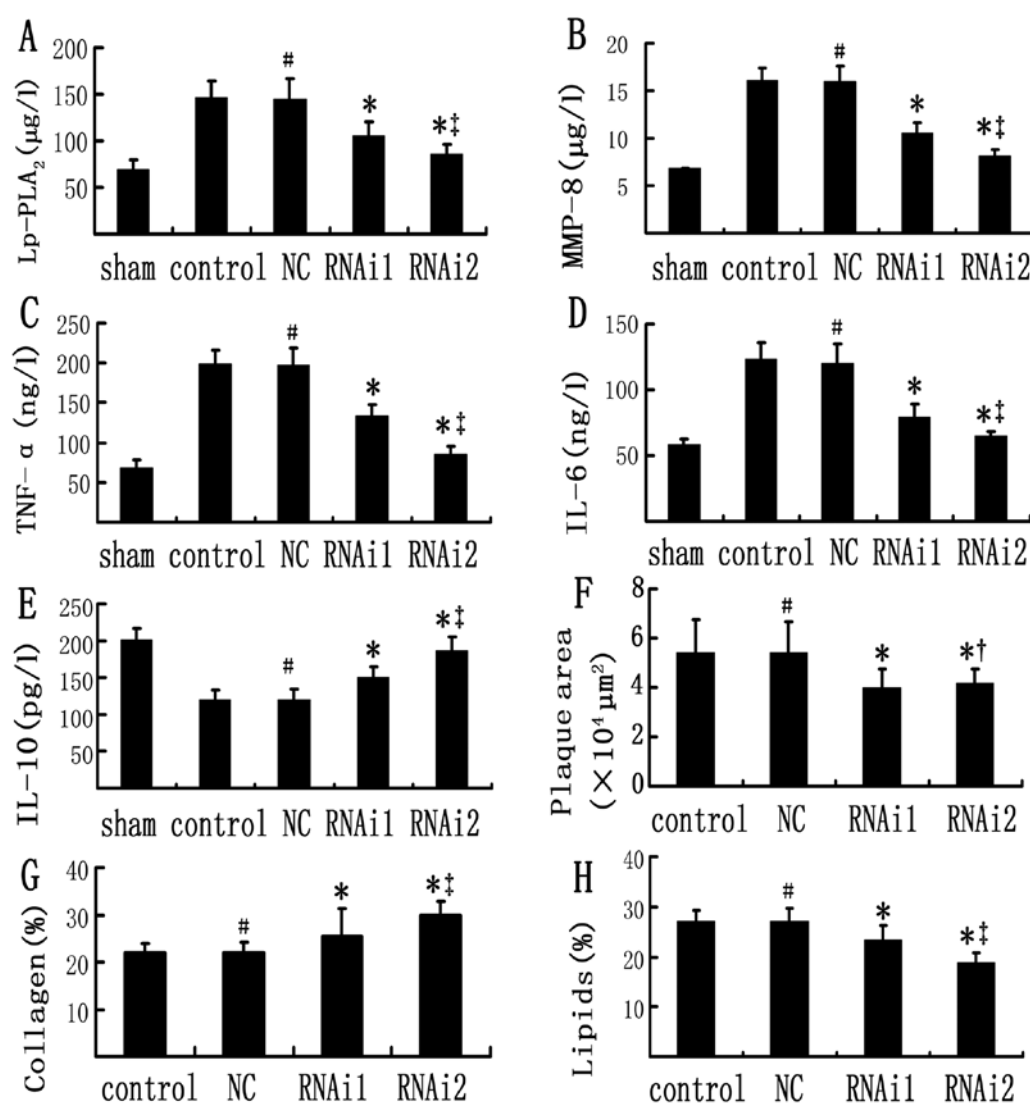


Figure 3. (A-E) Comparison of plasma inflammatory markers in the sham-operated, control, NC, RNAi1 and RNAi2 groups; (F-H) Comparison of plaque morphology in the different groups. Concentrations of (A) Lp-PLA₂, (B) MMP-8, (C) TNF-α, (D) IL-6 and (E) IL-10 were measured by ELISA at week 15. (F) Plaque area, (G) collagen content and (H) lipid content are shown for the control, NC, RNAi1 and RNAi2 groups. Data are expressed as the mean ± SD (n=18). No significant differences were found between control and NC groups. [#]P<0.05 vs. control and NC groups; ^{*}P<0.05 vs. RNAi1 group; [†]P>0.05 vs. RNAi1 group; [‡]P>0.05 vs. control group (one-way ANOVA). Lp-PLA₂, lipoprotein-associated phospholipase A₂; MMP-8, matrix metalloproteinase-8; TNF-α, tumor necrosis factor-α; RNAi, RNA interference; IL, interleukin.

fluorescence was visualized 4-6 weeks after transfection. Faint fluorescence was still visible 7 weeks after transfection. These results demonstrated efficient *in vivo* transfection of Lp-PLA₂ shRNA in the carotid plaques of the mice.

Effective silencing of Lp-PLA₂ expression by RNAi *in vivo*. At the end of the study, the plaques in the sham-operated group showed extremely low mRNA and protein expression of Lp-PLA₂ as well as the plasma concentration of Lp-PLA₂. The mRNA and protein expression of Lp-PLA₂, as well as the plasma concentration Lp-PLA₂ were significantly higher in the control and NC group compared with that in the sham-operated group. In the RNAi1 and RNAi2 groups, Lp-PLA₂ mRNA expression was reduced by 42.5 and 58.3% (both P<0.01), the Lp-PLA₂ protein level was decreased by 15.6 and 46.2% (both P<0.01) and the plasma concentration of Lp-PLA₂ was lowered by 28.2 and 40.8% (both P<0.05), respectively, compared to those in the control and NC groups (Figs. 3A and 5). In contrast, the control

group did not differ from the NC groups in Lp-PLA₂ expression. These results indicate that transluminal local administration of the lentivirus was more effective in silencing Lp-PLA₂ expression.

No effect of Lp-PLA₂ RNAi on body weight and plasma lipid profile. As expected, we observed no significant differences in the TC and TG levels among the 5 groups of mice. Additionally, the body weights of mice in all groups were not significantly different (Table I).

Lp-PLA₂ RNAi normalizes plasma inflammatory markers. Control and NC groups demonstrated a significant increase in the plasma concentration of pro-inflammatory cytokines MMP-8, TNF-α and IL-6 together with a reduction in the anti-inflammatory cytokine, IL-10, when compared with the sham-operated group (Fig. 3). These changes were partially reversed after RNAi. This was particularly evident in the

Table I. Body weight, plasma TC and TG levels among all groups.

	Sham	Control	NC	RNAi1	RNAi2
BW (g)	27.4±3.7	27.8±3.6	26.7±3.2	27.5±3.9	27.8±3.5
TC (mmol/l)	29.5±3.5	30.3±2.3	29.9±3.0	29.8±3.3	30.1±2.3
TG (mmol/l)	3.0±0.9	3.0±1.0	3.0±0.9	3.0±0.8	2.9±0.9

Data are reported as the mean ± SD of 18 animals. $P>0.05$ among all groups (one-way ANOVA). BW, body weight; TC, total cholesterol; TG, triglyceride; Sham, sham operated group; NC, negative control group; control, control group; RNAi1, RNAi group 1; RNAi2, RNAi group 2.

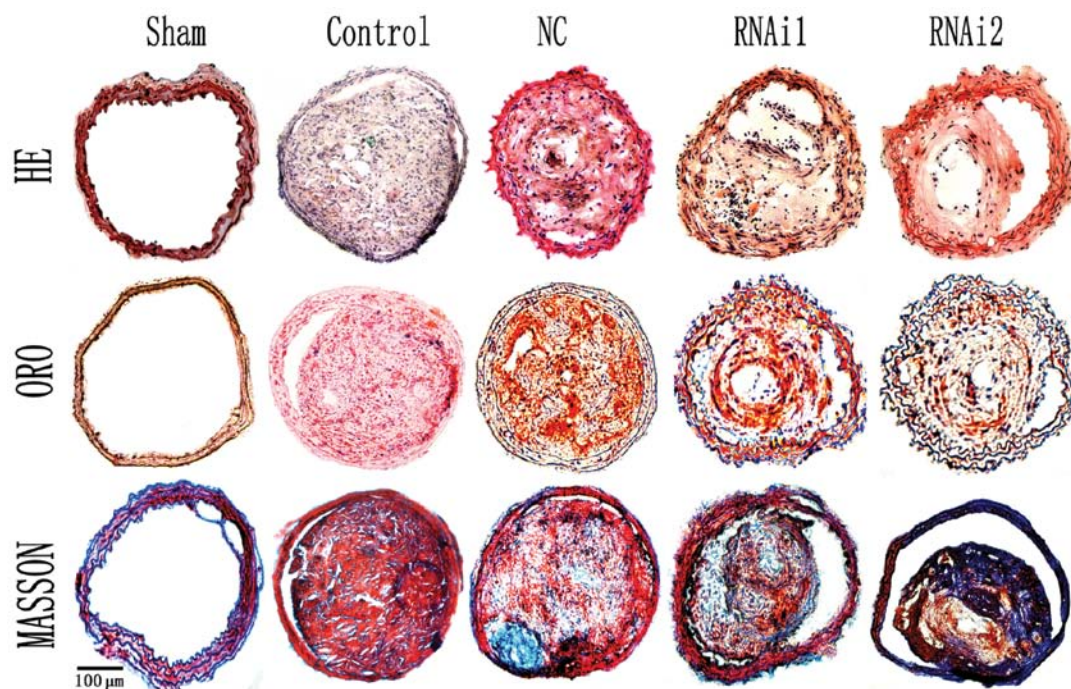


Figure 4. Representative microscopic images of atherosclerotic plaques in the sham-operated, control, NC, RNAi1 and RNAi2 groups. Cross-sections of atherosclerotic plaques in the different groups were stained for HE, ORO and Masson's trichrome. Magnification, x200. HE, hematoxylin and eosin; ORO, Oil Red O.

RNAi2 group, which showed significantly lower mRNA expression of pro-inflammatory cytokines than the control and NC groups did ($P<0.01$), reaching values similar to those observed in the sham-operated group.

Lp-PLA₂ RNAi attenuates the formation of atherosclerotic plaques. The cross-sectional plaque areas for the 2 RNAi groups were found to be significantly lower than those values in the control and NC groups at week 15 ($P<0.01$) (Fig. 4). As expected, no significant difference in plaque area was found between the control and NC groups. Interestingly, we also observed that the plaque area for the RNAi2 group was only moderately lower than that of the RNAi1 group, which was not statistically significant ($P>0.05$) (Figs. 3 and 4). This result suggested that delivery of Lp-PLA₂ shRNA either locally or systemically did not differentially affect plaque size.

The relative content of lipids and collagen in plaques was determined by histological staining (Figs. 4 and 5). The relative content of collagen in plaques of the control, NC, RNAi1, and

RNAi2 groups was 22.1, 22.0, 25.3 and 29.8%, respectively, and was significantly higher in the RNAi1 and RNAi2 groups ($P<0.01$). In comparison with the control and NC group, the relative increase in the collagen content in plaques of the RNAi1 and RNAi2 groups was 14.7 and 35.1%, respectively.

The relative content of lipids in plaques of the 4 groups was 27.1, 27.0, 23.5 and 18.9%, respectively, and was significantly lower in the RNAi1 and RNAi2 groups than that in the control and NC groups ($P<0.01$). The relative reduction in lipid content in plaques of the RNAi1 and RNAi2 groups was 26.2 and 30.1%, respectively, compared with the control and NC groups. In contrast, no significant difference in the content of lipids and collagen was found between the control and NC group. Note, that at the end of the study period, no atherosclerotic lesions were found in the left common carotid artery of the sham-operated group. Taken together, these data indicate that the 2 RNAi groups showed less lipid content and higher collagen content than the control and NC groups did. Although the 2 RNAi groups were both effective in

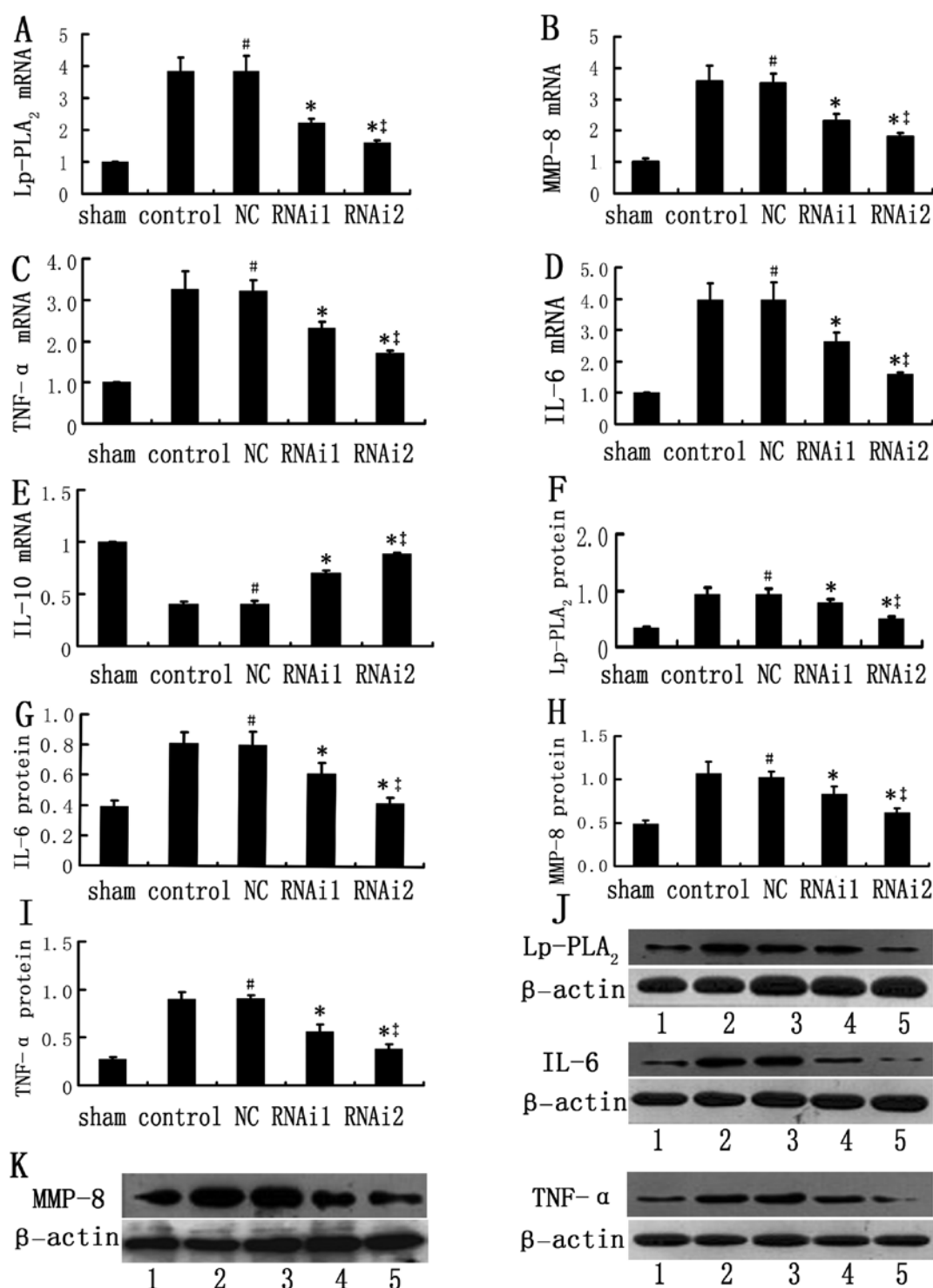


Figure 5. Expression of inflammatory genes in atherosclerotic plaques in the sham-operated, control, NC, RNAi1 and RNAi2 groups after harvesting of tissues at week 15. (A-E) mRNA expression of inflammatory genes. Relative mRNA expression of Lp-PLA₂ and inflammatory genes was measured by real time-PCR in the sham-operated, control, NC, RNAi1 and RNAi2 groups for 3 independent experiments. The expression levels of the target gene in the sham-operated group were considered as 1, its relative expression levels in other groups were presented as a ratio with that of the sham-operated group. (F-K) Densitometric analysis of protein expression of inflammatory genes and representative immunoblots. Accumulation of target protein was normalized against β-actin protein levels, determined as an internal control. Data are expressed as the mean ± SD (n=6). No significant differences were found between the control and NC groups. [#]P>0.05 vs. control group; ^{*}P<0.05 vs. control and NC groups; [†]P<0.05 vs. RNAi1 group (one-way ANOVA). Lane 1, sham-operated group; lane 2, control group; lane 3, negative control (NC) group; lane 4, RNAi1 group; lane 5, RNAi2 group. Lp-PLA₂, lipoprotein-associated phospholipase A₂; MMP-8, matrix metalloproteinase-8; TNF-α, tumor necrosis factor-α; RNAi, RNA interference; IL, interleukin.

attenuating atherosclerotic plaque formation and decreased plaque vulnerability, transluminal local delivery of Lp-PLA₂ shRNA exhibited enhanced improvement of plaque stability

than systemic administration. Plaque area and composition among all groups were not statistically significant at the time of transfection.

Effects of RNAi on the expression of inflammatory genes within the plaque. The plaques in the sham-operated group showed very low expression of MMP-8, TNF- α and IL-6 at the end of the study, in comparison, the expression of pro-inflammatory cytokines was sharply increased in the control and NC groups. Silencing of Lp-PLA₂ RNAi was able to reverse these increases; in the RNAi2 group there was significantly lower mRNA and protein expression of pro-inflammatory cytokines, as compared to the control and NC groups ($P < 0.05$), reaching values similar to those observed in sham-operated mice. In contrast to these factors which were increased in atherosclerotic plaques, mRNA expression of IL-10 was diminished in control and NC groups compared with the sham-operated group, and this effect was attenuated after RNAi. As expected, we observed no significant differences in the expression of inflammatory genes between the control and NC groups (Fig. 5).

Discussion

The major finding of the present study was that the mRNA and protein expression of Lp-PLA₂ can be effectively knocked down in carotid plaques of apolipoprotein E-deficient mice using lentiviral-mediated RNAi. This led to reduced local inflammatory cytokine expression, decreased lipid content in plaques, increased collagen content in plaques and reduced atherosclerotic plaque areas and vulnerability. Importantly, transluminal local delivery of Lp-PLA₂ was found to be more effective than systemic administration, thus providing a potential therapeutic approach for the treatment of atherosclerosis.

Atherosclerosis is a chronic inflammatory disease of the vascular wall (7). It is known that the inflammatory process contributes significantly to the initiation, progression and rupture of atherosclerotic plaques (1,14,15). Lp-PLA₂ produces 2 types of inflammatory mediators, LPC and oxNEFA, which trigger significant inflammatory responses, such as cell adhesion, inflammatory gene expression, and cell death (16,17). Both experimental and epidemiological studies have presented evidence that the circulating concentration of Lp-PLA₂ is associated with progression of atherosclerosis after adjusting for established risk factors (2,19,20). Given what is known about the actions of Lp-PLA₂ and the epidemiological data, Lp-PLA₂ provides an attractive target for assessing cardiovascular disease risk and a therapeutic target for interventions to reduce atherosclerosis. Previous studies have demonstrated that darapladib, a selective Lp-PLA₂ inhibitor, attenuated inflammation and necrotic core formation in animal models of atherosclerosis (21,22). However, darapladib did not reduce the primary end point of coronary plaque deformability, nor alter the plasma hs-CRP concentration in a phase II clinical study (23,24). In summary, experimental and epidemiological evidence remains equivocal concerning the potentially pro-atherogenic and anti-atherogenic effects of Lp-PLA₂ inhibition by darapladib. RNAi is a clinically feasible method with which to downregulate the expression of target genes efficiently and selectively (18). In the present study, lentiviral-mediated Lp-PLA₂ RNAi was used as a therapeutic approach for atherosclerosis. Traditional concepts of inflammation in atherosclerosis are regarded as an 'inside-out' responses, holding the central tenet that inflammatory responses are initiated at the luminal surface, and later propa-

gate outward toward the adventitia. Therefore, we proceeded with transfection using a transluminal approach. The efficacy of lentiviral-mediated RNAi was confirmed by an observed decrease in the mRNA and protein expression of Lp-PLA₂ and the observation of GFP fluorescence in the plaques. This effect was associated with a reversal of the observed increase in the expression of pro-inflammatory cytokines (MMP-8, TNF- α and IL-6), and an attenuation of the decrease in expression of anti-inflammatory cytokines (IL-10), as well as, decreased plaque content of lipids, increased plaque content of collagen, and finally lowered atherosclerotic plaque area and vulnerability of the plaques. We denied the possibility that the beneficial effects observed in the RNAi group were caused by nonspecific immune stimulation induced by transfection since no significant effect was found between the control and NC groups.

Several lines of evidence suggest that the precise role of Lp-PLA₂ in atherosclerosis in mice, with a lipoprotein profile different to humans, is controversial, with previous studies proposing seemingly contradictory anti- and pro-atherogenic functions. Lp-PLA₂ is an enzyme mainly associated with HDL in mice and LDL in humans (4). A study by Tellis and Tselepis (25) suggested that the role of Lp-PLA₂ in atherosclerosis may depend on its lipoprotein carrier in plasma, and that HDL-associated Lp-PLA₂ contributes to the reduction of atherosclerosis, whereas LDL-associated Lp-PLA₂ stimulates this process. Nevertheless, other research has demonstrated that increased plasma Lp-PLA₂ is associated with susceptibility to atherosclerosis in mice (26), and that patients with coronary heart disease exhibit reduced LDL-Lp-PLA₂ mass and catalytic efficiency, suggesting a diminished ability to degrade pro-inflammatory phospholipids. Moreover, considerable evidence has been obtained for the pro-atherogenic roles of Lp-PLA₂ *in vitro* and *in vivo* (26-28).

It was initially thought that Lp-PLA₂ exerts an anti-atherogenic and anti-inflammatory effect by hydrolyzing and inactivating platelet activating factor (PAF), a well-known pro-inflammatory factor that contributes to inflammation and atherosclerosis (29). Despite this, individuals with reduced levels of Lp-PLA₂ activity do not display rampant inflammatory responses anticipated from uncontrolled PAF accumulation, and acute bronchoconstriction to inhaled PAF does not vary in these individuals (24,30,31). Notably, responsiveness to PAF is not altered in Japanese subjects with a genetic variant in Lp-PLA₂ (Val276Phe) that results in absence of the circulating enzyme (32,33). In addition, clinical trials failed to show measurable benefit of recombinant human Lp-PLA₂ (also termed PAFAH) in patients with asthma or septic shock (34,35). Furthermore, a recent report by Liu *et al* (24) indicated that circulating PAF is primarily cleared by PAF receptor-independent transport, rather than intravascular hydrolysis by PAFAH. In summary, we find no evidence that Lp-PLA₂ hydrolyzes PAF *in vivo*.

In the present study, we observed a marked effect of RNAi on circulating inflammatory markers. These results are in agreement with previous studies showing that atherosclerosis is an inflammatory process (5,14,19,36). LPC, the hydrolyzing product of Lp-PLA₂, has been shown to contribute to oxidative stress in macrophages and their tissue accumulation. Macrophages are the most significant source of Lp-PLA₂ in

the vascular wall. By virtue of these processes, Lp-PLA₂ is involved in a positive-feedback loop of inflammation and atherosclerosis. Macrophages are the main source of pro-inflammatory cytokines, such as MMP-8, IL-6 and TNF- α . High levels of Lp-PLA₂ and pro-inflammatory cytokines may therefore favor the development of vulnerable plaques. Our study suggests that Lp-PLA₂ RNAi decreased the expression of pro-inflammatory cytokines, thereby playing an anti-atherogenic and anti-inflammatory role. A possible explanation for this beneficial effect may be that Lp-PLA₂ RNAi attenuated the accumulation of macrophages in atherosclerotic plaques, as indicated by our cell experiments revealing that RNAi attenuated the expression of MCP-1, IL-6 and Lp-PLA₂ evoked by oxLDL. MMP-8 possesses proteolytic activity on several matrix proteins particularly type I collagen and on various non-matrix proteins (37). The RNAi groups manifested diminished MMP-8 expression and vulnerability of the plaques, which was in agreement with a previous study indicating that atherosclerotic lesions in MMP-8-deficient mice had increased collagen content (37). Recent studies suggest that IL-10 may be a key mediator of vascular protection in atherosclerosis (38). A major role for IL-10 is to inhibit expression of pro-inflammatory cytokines including MMP-8, IL-6 and TNF- α (37,39,40). These pro-inflammatory cytokines are also known to contribute to vascular inflammation, plaque destabilization and thrombosis (37). In the present study, higher levels of MMP-8, TNF- α and IL-6 were found in the carotid arteries of control and NC mice, and this effect was almost completely abolished by RNAi. Additionally, we observed a marked increase in the collagen content of plaques in the RNAi groups, suggesting increased plaque stability. This effect was more pronounced in the group receiving trans-luminal local transfection of the lentivirus, supporting the idea that transluminal local delivery of Lp-PLA₂ shRNA was superior to systemic administration in stabilizing atherosclerotic plaques. Collectively, our results suggest that Lp-PLA₂ RNAi decreased the expression of pro-inflammatory cytokines, as well as it increased the expression of anti-inflammatory cytokines, thereby playing an anti-atherogenic and anti-inflammatory role in the stabilization of vulnerable plaques.

Lp-PLA₂ is upregulated in atherosclerotic plaques and macrophages undergoing apoptosis within the necrotic core and fibrous cap of vulnerable and ruptured plaques, but not within stable lesions. As Lp-PLA₂ predominantly existed in advanced plaques, it may play an important role in advanced lesions and the determination of plaque instability, but not at earlier stages of atherogenesis. Therefore, the duration of the present investigation was 7 weeks, which was longer than the 3-week duration used in the study of Quarck *et al* (29).

The present study had several limitations. Firstly, we only measured the plasma concentration of Lp-PLA₂ at the end of the investigation, which may not reflect the true activity of Lp-PLA₂ over time. Secondly, our data revealed no difference in plaque area between the 2 RNAi groups. This may be due to the relatively small number of animals undergoing histological analysis, thus we cannot exclude the possibility that the lack of difference was due to low statistical power. Further research will be needed to clarify these details.

In summary, our study demonstrated that lentiviral-mediated RNAi was effective in knocking down Lp-PLA₂

expression in apolipoprotein E-deficient mice, which resulted in reduced inflammatory gene expression, diminished plaque area, decreased lipid content, increased collagen content and reduced plaque vulnerability, independent of the plasma lipoprotein profile. In addition, transluminal local delivery of Lp-PLA₂ shRNA was superior to systemic administration in stabilizing atherosclerotic plaques.

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