ORIGINAL RESEARCH

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Amelioration of atherosclerosis in apolipoprotein E-deficient mice by inhibition of lipoprotein-associated phospholipase A₂

Abstract

Purpose: Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is involved in the pathogenesis of atherosclerosis, especially in advanced plaques. In the present study, the abilities of darapladib, a selective Lp-PLA₂ inhibitor, and lentivirus-mediated Lp-PLA₂ silencing on inflammation and atherosclerosis in apolipoprotein E-deficient mice were compared.

Methods: Apolipoprotein E-deficient mice were fed on a high-fat diet and a constrictive collar was placed around the left carotid artery to induce plaque formation. The mice were randomly divided into control, negative control (NC), darapladib and RNA interference (RNAi) groups. Eight weeks after surgery, lentivirus-mediated RNAi construct or darapladib were used to decrease the expression of Lp-PLA₂. Plaques were collected five weeks later for histological analysis. Inflammatory gene expression in the atherosclerotic lesions were then determined at the mRNA and protein level.

Results: The expression of pro-inflammatory cytokines was significantly reduced in the treatment group, compared to nontreatment group, whereas the plasma concentration of anti-inflammatory cytokines increased markedly. Moreover, our results demonstrated a significant reduction in plaque lipid content, as well as a rise in collagen content following Lp-PLA₂ inhibition. Interestingly, when comparing the two methods of Lp-PLA₂ inhibition, animals treated with Lp-PLA₂ RNAi were found to exhibit lower plaque areas and enhanced improvement of plaque stability as compared with animals treated with darapladib. Darapladib had no attenuating effect on atherosclerotic plaque area. These therapeutic effects were independent of plasma lipoprotein levels.

Conclusions: Lp-PLA₂ inhibition by darapladib or lentivirus-mediated RNAi ameliorated inflammation and atherosclerosis in apolipoprotein E-deficient mice. The effect was more prominent in the RNAi group.

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Despite major advances in treatment of atherosclerosis, a large number of victims of the disease, who are apparently healthy, die suddenly without prior symptoms. This observation led us to identify possible novel therapeutic targets in the treatment of atherosclerosis. An interesting target for cardiovascular drug development is lipoprotein-associated phospholipase A₂ (Lp-PLA₂, also termed platelet-activating factor acetylhydrolase, PAFAH). Lp-PLA₂ is an enzyme that plays an atherogenic role by hydrolyzing platelet activating factor (PAF) and oxidized phospholipids, resulting in the generation of two bioactive lipid mediators, lysophosphatidylcholine (LPC) and oxidized non-esterified fatty acid (oxNEFA), both of which play key roles in atherosclerosis [1-5]. Epidemiological studies have supported Lp-PLA₂ as a cardiovascular risk marker independent of traditional risk factors [6-9].

Given its biology and epidemiology, Lp-PLA₂ provides an attractive target for intervention to reduce atherosclerosis. In a previous study, darapladib, a selective Lp-PLA₂ inhibitor, decreased intraplaque LPC content and reduced the necrotic core in animal models of atherosclerosis [10]. Chronic suppression of circulating Lp-PLA₂ activity with darapladib did not reduce human plaque volume nor alter plasma hs-CRP concentration in a phase II clinical study [11,12]. In summary, experimental and epidemiological evidences are not equivocal about the potentially proatherogenic and antiatherogenic effects of darapladib [13].

RNA interference (RNAi) has been shown to be quite efficacious in silencing target genes in both dividing and non-dividing cells [14]; however, no studies have investigated the effects of Lp-PLA₂ silencing on the progression of atherosclerosis in mice models. In the present study, the effects of darapladib and lentivirus-mediated Lp-PLA₂ RNAi on the progression of atherosclerosis in apolipoprotein E deficient mice were compared.

Materials and Methods

Lp-PLA₂ RNAi lentiviral vectors

Lp-PLA₂ RNAi and negative control (NC) lentivirus expressing non-targeting sequences were used (Shanghai Genepharma Co., Shanghai, China). The target sequence for Lp-PLA₂ RNAi was 5'-GCAAGCTGGAATTCTCCTTTG-3'. A scrambled shRNA sequence (5'-TTCTCCGAACGTGTCACGT-3') served as NC. Vectors were made as previously described [15, 16]. The titers averaged to 1×10^9 TU (transduction units)/ml.

Cell culture

The RAW264.7 mouse macrophage cell line was routinely cultured in DMEM. When cells had grown to 90% confluence, the Lp-PLA₂ RNAi lentiviruses and lenti-scrambled-shRNA were then used to transfect RAW264.7 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 up-regulated 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 µg/ml of oxLDL stimulation. Therefore, the cells were pretreated with 60 µg/ml oxLDL. Next, the effects of RNAi and darapladib on the expression of Lp-PLA₂ and matrix metalloproteinase-8 (MMP-8) were investigated using quantitative real-time PCR. Non-lentivirus- and lentivirus-containing NC shRNA transfection served as controls.

Animal protocol

Ninety six 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 anaesthesia with an intra-peritoneal injection of pentobarbital sodium (30-50 mg/kg) [17]. In brief, the common carotid arteries were dissected and a constrictive silastic collar (0.30 mm) was placed on the left common carotid artery by placement of three circumferential silk ties in all mice. Subsequently, the entry wound was closed and the animals were returned to their cage for recovery from anaesthesia. Eight weeks after surgery, the carotid collars were removed under anaesthesia. The mice were randomly allocated to either the treatment group or non-treatment group (n= 48). Treatment group mice were then randomly distributed into two subgroups of 24 animals each: the darapladib group, in which darapladib was administered in the following 5 weeks at a dose of 50 mg/kg/day and the RNAi group, in which 10^8 TU (100 µl) of viral suspension was injected into the tail vein of the mice. Non-treatment group mice were also randomly distributed into two subgroups of 24 animals each: the control group had their collars removed without drug administration; the NC group received 10^8 TU (100 µl) of NC lentivirus injected into the tail vein of the mice [18]. 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).

TABLE 1. Primers used for real time-PCR

Gene	Primer	Product size (bp)
Lp-PLA ₂	Sense: 5'-CCAGAGATTCAGATGTGGAGTT-3'	104
	Antisense: 5'-TGGCAGAGTTGATAAAGAGGAG-3'	
IL-6	Sense: 5'-ACAACCACGGCCTTCCCTACTT-3'	140
	Antisense: 5'-TTTCTCATTTCCACGATTTCCC-3'	
IL-10	Sense: 5'-CTGGACAACATACTGCTAACCG-3'	148
	Antisense: 5'-TCAAATGCTCCTTGATTTCTGG-3'	
MMP-8	Sense: 5'-GCCTGACTCTGGTGATTTCTTG-3'	200
	Antisense: 5'-TGTTGATGTCTGCTTCTCCCTG-3'	
β -actin	Sense: 5'-GCTATGCTCTCCCTCACGCCAT-3'	130
	Antisense: 5'-TCACGCACGATTTCCCTCTCAG-3'	

Lp-PLA₂: Lipoprotein-associated phospholipase A₂; IL: interleukin; MMP-8: Matrix Metalloproteinase-8;

Histological analysis

All mice were sacrificed at week 13. Mice were perfused with PBS via the left ventricle. The carotid artery was cross-sectioned and stained with hematoxylin and eosin (HE); plaque collagen and lipid deposition were identified using Masson's trichrome and oil red O (ORO) staining, respectively.

RNA extraction and real time-PCR

Total RNA was extracted with Trizol reagent. 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, USA). The transcript amount of β -actin was used as an internal control. Quantitative values were obtained from the threshold cycle (C_t). The specific primers are given in Table 1. The results were analyzed by the $2^{-\Delta\Delta C_t}$ method.

Western blot analysis

Carotid arteries were collected and lysed with protease inhibitor in $1 \times$ lysis buffer on ice for 10 to 15 minutes. Homogenates were centrifuged at 12,000 g for 20 min on ice, and supernatants were collected and protein content was quantified using the BCA method, and then SDS-PAGE electrophoresis was performed. Proteins were transferred to PVDF membranes, membranes were blocked with 5% non-fat milk and incubated overnight with primary antibodies against Lp-PLA₂,

MMP-8 (Abcam, Cambridge, UK), IL-10, IL-6 and β -actin (Zhongshan, 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. Quantitative analysis was performed by ImageJ software and expression level was normalized to β -actin.

Plasma lipid and biological analysis

Plasma concentration of Lp-PLA₂, IL-10, MMP-8, IL-6, PAF, LPC and plasma lipoprotein level were measured using commercial kits (CoWin-Bio-science, Beijing, China) following the manufacturer's recommendation.

Statistical analysis

Data are presented as mean values $\pm$ standard deviation (SD). Comparisons were made using one-way analysis of variance, followed by the Student-Newman-Keuls (SNK) post hoc tests for significance. All analyses involved the use of SPSS 16.0 statistical analysis software (SPSS, Inc., Chicago, USA). $P < 0.05$ was considered statistically significant.

FIGURE 1. Effects of RNAi and darapladib *in vitro*. (A)-(C) are real-time PCR quantification of Lp-PLA₂ and MMP-8 mRNA expression in RAW 264.7 cells; After 60 μg/ml of oxLDL pretreatment, the expression of Lp-PLA₂ increased sharply. (A): Mouse RAW264.7 cells were untransfected or transfected with NC LV or Lp-PLA₂ RNAi LV (MOI = 50) to determine their silencing efficiency. Compared with cells treated with oxLDL alone, cells treated with virus suspension demonstrated significant inhibition of Lp-PLA₂ expression. (B): RNAi inhibited the augmentation of MMP-8 induced by oxLDL. (C): Darapladib decreased the mRNA expression of IL-8 in a dose dependent manner, the effect of the Lp-PLA₂ inhibitor reached the platform stage after 100μg/ml of darapladib administration. Symbols - and + indicate the absence and presence of oxLDL, lentiviruse or darapladib. * P < 0.05 vs. control groups; ▲ P > 0.05 vs. control group; † P > 0.05 vs. darapladib 100 μg/ml.

FIGURE 2: Effects of darapladib and RNAi on the expression of Lp-PLA₂ and plasma concentration of inflammatory markers *in vivo*. (A)-(C) are plasma concentration, Western blotting and real-time PCR analysis of Lp-PLA₂ expression in the control, NC, darapladib and RNAi groups. (D)-(H) are plasma concentrations of IL-6, MMP-8, LPC, IL-10 and PAF in the control, NC, darapladib and RNAi groups. * P < 0.05 vs. control and NC groups; † P < 0.05 vs. darapladib group; ‡ P > 0.05 vs. darapladib group; ▲ P > 0.05 vs. control and NC groups. Lp-PLA₂: Lipoprotein-associated phospholipase A₂; MMP-8: Matrix Metalloproteinase-8; RNAi: RNA interference; IL: interleukin; LPC: lyso-phosphatidylcholine; PAF: platelet activating factor.

Results

Effects of darapladib and Lp-PLA₂ RNAi *in vitro*

After 60 μg/ml of oxLDL pretreatment, the expression of Lp-PLA₂ increased sharply. Mouse RAW264.7 cells were then untransfected or transfected with NC lentivirus or Lp-PLA₂ RNAi lentivirus (MOI = 50) to determine their silencing efficiency. Our results demonstrated that Lp-PLA₂ RNAi or darapladib inhibited the augmentation of Lp-PLA₂ and MMP-8

induced by oxLDL. In addition, darapladib decreased the mRNA expression of IL-8 in a dose-dependent manner (Figure 1).

Effects of darapladib and Lp-PLA₂ RNAi on the expression of Lp-PLA₂ *in vivo*

Lp-PLA₂ inhibition decreased the expression of Lp-PLA₂ in the treatment group. In addition, mRNA and protein expression of Lp-PLA₂ were significantly lower in the RNAi group

TABLE 2. Body weight, plasma TC and TG levels among all groups

	BW (g)	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
Control	26.1 ± 2.8	29.2 ± 3.1	2.9 ± 0.8	3.4±0.4	29.7±4.0
NC	26.4 ± 3.5	29.6 ± 2.2	3.1 ± 0.9	3.3±0.4	29.8±3.6
Darapladib	26.4 ± 3.0	29.8 ± 2.7	2.9 ± 0.8	3.4±0.3	30.1±3.2
RNAi	26.1 ± 2.8	29.8 ± 3.7	3.0 ± 0.8	3.4±0.5	30.4±4.8

Data are reported as the mean ± SD. $P > 0.05$ among all groups. BW=body weight; TC=total cholesterol; TG=triglyceride; HDL-C=high-density lipoprotein cholesterol; LDL-C=low-density lipoprotein cholesterol; NC=negative control group. RNAi= RNA interference group.

compared with the darapladib group, although the plasma concentrations of Lp-PLA₂ were similar between the two groups (Figure 2 and 5G). As expected, the control group did not differ from the NC group in Lp-PLA₂ expression.

Lp-PLA₂ inhibition has no significant effects on body weight and plasma lipid profile

As expected, no significant differences in the TC, TG, HDL-C and LDL-C levels were observed among all groups. Additionally, the body weights of mice were not significantly different (Table 2).

Effects of Lp-PLA₂ inhibition on plasma concentration of inflammatory markers

The treatment group had lower plasma concentrations of pro-inflammatory cytokines (IL-6, LPC and MMP-8) together with increased concentrations of anti-inflammatory cytokine (IL-10) when compared with the non-treatment group. In contrast, the plasma levels of PAF did not differ among the four subgroups. In addition, no difference in plasma concentration of inflammatory markers was found between the darapladib group and the RNAi group (Figure 2). As expected, the control group did not differ from the NC group in these markers.

Effects of Lp-PLA₂ inhibition on the morphology of plaques

The relative content of collagen in plaques of the control, NC, darapladib and RNAi groups was 22.5, 22.4, 33.6 and 36.7%, respectively, and was significantly higher in the RNAi group than that in the darapladib group ($P < 0.01$). The lipid content in the plaques of the control, NC, darapladib and RNAi groups were 27.6, 27.4, 19.3 and 15.3%, respectively, and was significantly lower in the RNAi group than that in the darapladib group ($P < 0.01$). Thus, the treatment group showed lower

lipid content and higher collagen content than the non-treatment group (Figure 3, 4).

Fibrous cap thickness was significantly higher in the RNAi group than that in the darapladib and non-treatment groups ($P < 0.01$). Cross-sectional plaque area was significantly lower in the RNAi group than that in the darapladib and non-treatment groups ($P < 0.01$). Interestingly, the plaque area for the darapladib group was not significantly different from that of the non-treatment group ($P > 0.05$). Taken together, these data indicate that the RNAi group showed less lipid content, lower plaque area, higher collagen content and fibrous cap thickness than the darapladib and non-treatment groups. Although the two treatments were both effective in stabilizing atherosclerotic plaques, RNAi group exhibited reduced plaque area and enhanced plaque stability as compared to the darapladib group. Darapladib had no effect on attenuating the atherosclerotic plaque area, although darapladib administration improved the stability of the plaque. Plaque area and composition among all groups were not statistically significant at week 8.

Effects of Lp-PLA₂ inhibition on the expression of inflammatory genes within the plaque

Lp-PLA₂ inhibition decreased the expression of pro-inflammatory cytokines (MMP-8 and IL-6) and increased the expression of anti-inflammatory cytokines (IL-10) in atherosclerotic plaques. This effect was greater with RNAi treatment. As expected, no significant differences in the expression of inflammatory genes were observed between control and NC groups (Figure 5).

FIGURE 3. Histopathological staining showing plaque composition in the control, NC, darapladib and RNAi groups. Cross-sections of atherosclerotic plaques were stained for HE, ORO and Masson's trichrome, respectively. Magnification, 200x.

FIGURE 4. Comparison of plaque morphology. Data for control, NC, darapladib and RNAi groups are shown for lipids content (A), collagen content (B), fibrous cap thickness (C) and the plaque area (D). * $P < 0.05$ vs. control and NC groups; ▲ $P > 0.05$ vs. control and NC groups; † $P < 0.05$ vs. darapladib group.

FIGURE 5. Expression of inflammatory genes in atherosclerotic plaques in the control, NC, darapladib and RNAi groups of mice. (A) IL-6 protein; (B) MMP-8 protein; (C) IL-10 protein; (D) IL-6 mRNA; (E) MMP-8 mRNA; (F) IL-10 mRNA. (G) Representative Western blots used for quantification. * $P < 0.05$ vs. control and NC groups; † $P < 0.05$ vs. darapladib group. 1: control group; 2: negative control (NC) group; 3: darapladib group; 4: RNAi group; MMP-8: Matrix Metalloproteinase-8; RNAi: RNA interference; IL: interleukin.

Discussion

In the present study, the effects of Lp-PLA₂ inhibition by darapladib or lentivirus-mediated RNAi on the progression of atherosclerosis and associated inflammatory process following collar induced atherosclerosis in apolipoprotein E deficient mice were investigated. Both RNAi and darapladib ameliorated atherosclerosis and reduced the expression of pro-inflammatory cytokines, suggesting that Lp-PLA₂ inhibition can exert a potentially therapeutic effect. In addition, the beneficial effects were more pronounced in the RNAi group, indicating that Lp-PLA₂ RNAi was superior to darapladib in ameliorating atherosclerosis.

It is clear that atherosclerosis is not only a lipid disease, but a complex, intertwined, inflammatory disease [19, 20]. This possibly explains why, despite state of the art therapies, significant residual risk of morbidity and mortality from atherosclerosis still persists [21]. This led us to identify novel risk factors as well as mediators of atherosclerosis. Lp-PLA₂ is responsible for the metabolism of oxidized phospholipids to the proatherogenic mediators LPC and oxNEFA [11], both of which contribute to inflammation and atherosclerosis [3, 11]. Given its biology and epidemiology, darapladib provides an attractive target for intervention to reduce atherosclerosis. Previous studies demonstrated that darapladib decreased intra-plaque LPC content, attenuated the expression of inflammatory genes, and reduced necrotic core formation in animal models of atherosclerosis [10, 22]; however, darapladib did not

reduce human plaque volume in a phase II clinical study [11, 12]. In summary, experimental and epidemiological evidence is not equivocal about the effects of darapladib [13,18, 23]. RNAi has been shown to be quite efficacious in silencing target genes in both dividing and non-dividing cells [14], as such we employed the two methods (darapladib and RNAi) and compared the distinct mechanisms involved in atherosclerosis. Our data indicate that darapladib and lentivirus-mediated Lp-PLA₂ silencing is equally effective in decreasing the concentration of pro-inflammatory cytokines in plasma. Moreover, the reduction in the lesion expression of Lp-PLA₂ and pro-inflammatory cytokines is more prominent in the RNAi group than that in the darapladib group, supporting the idea that RNAi was superior to darapladib in attenuating the expression of Lp-PLA₂ and pro-inflammatory cytokines in plaques. Additionally, these beneficial effects observed in the treatment groups were associated with decreased lipid content, increased collagen content and increased fibrous cap thickness. This effect was more pronounced in the RNAi group, supporting the idea that Lp-PLA₂ RNAi was superior to darapladib in stabilizing atherosclerotic plaques. Furthermore, the plaque area was reduced only in the RNAi group, suggesting that plaque area is more closely related to the concentration of pro-inflammatory cytokines in plaques than to the concentration of pro-inflammatory cytokines in plasma. No significant differences in body weight and plasma lipoprotein levels were found among the groups, indicating that RNAi was safe and the therapeutic effect was independent of the plasma lipoprotein profile.

Lp-PLA₂ is present mainly on LDL in humans, whereas HDL is the preferred carrier in mice. Several lines of evidences suggest that the mouse model, with a lipoprotein profile different from humans, was inadequate for studying the effects of Lp-PLA₂ inhibition *in vivo* because HDL-associated Lp-PLA₂ contributed to the reduction of atherosclerosis, whereas LDL-associated Lp-PLA₂ stimulated this process [6]. Controversy exists regarding this process, as increased plasma Lp-PLA₂ has also been associated with susceptibility to atherosclerosis in mice, and coronary heart disease patient exhibit reduced LDL-Lp-PLA₂ mass and catalytic efficiency, suggesting a diminished ability to degrade pro-inflammatory phospholipids [24, 25]. Indeed the biological role of Lp-PLA₂ is controversial since the anti- or proatherogenic functions of this enzyme have been proposed. Lp-PLA₂ might be antiatherogenic if it hydrolyzes PAF, a well-known proinflammatory factor that contributes to inflammation and atherosclerosis [22, 26]. Despite this, individuals with reduced levels of Lp-PLA₂ activity do not display the rampant inflammatory responses anticipated from uncontrolled PAF accumulation [12], and the acute bronchoconstrictive response to inhaled PAF does not vary in these individuals [12, 27, 28]. Moreover, there is no evidence that Lp-PLA₂ hydrolyzes PAF *in vivo*. In a recent study by Hu *et al.*, darapladib did not change plasma PAF levels [22]. Similarly, intravenous administration of human recombinant Lp-PLA₂ failed to alter PAF-mediated responses or mortality in patients with asthma or septic shock [27, 29]. Finally, a recent report by Liu *et al.* indicated that circulating PAF is primarily cleared by PAF receptor-independent transport, rather than by intravascular hydrolysis by Lp-PLA₂ *in vivo*. Indeed, no evidence that Lp-PLA₂ hydrolyze PAF *in vivo* was found in this study. Most research favors the pro-inflammatory and pro-atherogenic role of Lp-PLA₂ [24, 25, 30, 31].

In the present investigation we observed marked effect of Lp-PLA₂ RNAi on the expression of inflammatory cytokines. Atherosclerosis is an inflammatory disease [19]. LPC, the hydrolyzing product of Lp-PLA₂, has been shown to contribute to inflammation and macrophages accumulation in the lesion. Macrophages are the most significant source of Lp-PLA₂, MMP-8 and IL-6 in the lesion. By virtue of these processes, Lp-PLA₂ is involved in a positive-feedback loop of inflammation and atherosclerosis. MMP-8 and IL-6 are the key multifunctional cytokines which mediate inflammatory responses in atherosclerosis [32, 33, 34]. These pro-inflammatory cytokines are known to contribute to vascular inflammation and plaque destabilization. In the present work, higher levels of MMP-8 were found in the carotid plaques of control and NC groups, and this effect was decreased significantly by Lp-PLA₂ RNAi.

In addition, our cell experiments demonstrated that Lp-PLA₂ RNAi inhibited the expression of MMP-8 evoked by ox-LDL. These results were in line with a previous study indicating that atherosclerotic lesions in MMP8-deficient mice had significantly fewer macrophages but increased collagen content [32]. Collectively, our results suggest that Lp-PLA₂ RNAi decreased the expression of pro-inflammatory cytokines, as well as increased the expression of anti-inflammatory cytokines (IL-10), thereby played an anti-atherogenic and anti-inflammatory role in the stabilization of vulnerable plaques.

Lp-PLA₂ RNAi ameliorated atherosclerosis in apolipoprotein E-deficient mice. Our results were in accordance with previous studies which associated increased Lp-PLA₂ levels with inflammation and atherosclerosis; however, our results differed from Quarck *et al.* who reported that adenovirus-mediated Lp-PLA₂ gene transfer prevented injury-induced neointima formation in mice [18]. These contradictions may be due to the varied penetrance of pro-atherogenic stimuli at different arterial sites. In addition, distinct temporal roles for Lp-PLA₂ during lesion pathogenesis cannot be ruled out. Lp-PLA₂ is up-regulated in macrophages within vulnerable and ruptured plaques, but not within stable lesions [35, 36]. As Lp-PLA₂ predominantly exists 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. For this reason, the duration of the current investigation was five weeks, which is longer than the three weeks in the study by Quarck *et al.* [18].

Lentiviral vectors provide a HIV-1-based self-inactivating lentivirus for gene therapy. A significant number of safety features were designed to enhance its bio-safety and to minimize its relation to human HIV-1 virus. Further research is still needed to clarify the bio-safety of the lentivirus vectors.

Limitations of the study should be considered. To begin with, the plasma concentration of Lp-PLA₂ were measured only at the end of the investigation, which may not reflect the variation of Lp-PLA₂ over time. Secondly, our data demonstrated that darapladib group exhibited enhanced stabilization of carotid plaques than the control group, but the plaque area between the two groups was similar. Possible explanation for this phenomenon might be the dose of darapladib used in the present study was relatively small or that the duration of this study was not long enough to detect these variation. Further research is needed to clarify these details.

In conclusion, Lp-PLA₂ inhibition was effective in ameliorating atherosclerosis in apolipoprotein E-deficient mice, independent of the plasma lipoprotein profile. In addition, lentivirus-mediated Lp-PLA₂ RNAi was superior to darapladib

in decreasing the expression of pro-inflammatory gene, increasing the expression of anti-inflammatory gene and attenuating atherosclerosis. Our work indicated that Lp-PLA₂ RNAi provided a novel therapeutic approach to the treatment of atherosclerosis.

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List of Abbreviations

Lp-PLA ₂	Lipoprotein associated phospholipase A ₂
RNAi	RNA interference
NC	negative control
PAF	platelet activating factor
LPC	lysophosphatidylcholine
oxNEFA	non-esterified fatty acid
TU	transduction units
ORO	oil red O
TC	total cholesterol
TG	triglyceride
HDL-C	high-density lipoprotein cholesterol
LDL-C	low-density lipoprotein cholesterol
IL-6	interleukin-6
MMP-8	Matrix Metalloproteinase-8

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