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Original Article

The inhibition of lipoprotein-associated phospholipase A2 exerts beneficial effects against atherosclerosis in LDLR-deficient mice

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Aim: To investigate the effects of darapladib, a specific inhibitor of lipoprotein-associated phospholipase A2 (lp-PLA2), on inflammation and atherosclerotic formation in the low density lipoprotein receptor (LDLR)-deficient mice.

Methods: Six-week-old LDLR-deficient mice were fed an atherogenic high-fat diet for 17 weeks and then randomly divided into two groups. One group was administered darapladib (50 mg·kg^{-1}·d^{-1}; *po*) for 6 weeks. The other group was administered saline as a control. Serum lipid levels were measured using the corresponding kits, and three inflammatory markers — interleukin-6 (IL-6), C reactive protein (hs-CRP), and platelet activating factor (PAF) — were determined using ELISA. Atherosclerotic plaque areas were stained with Sudan IV, and inflammatory gene expression at the lesions was evaluated using quantitative real-time PCR.

Results: The body weight and serum lipid level between the two groups were similar at the end of the dietary period. The serum lp-PLA2 activity, hs-CRP and IL-6 levels, however, were significantly reduced in the darpladib group. The inhibition of lp-PLA2 did not alter the serum PAF level. Furthermore, the plaque area, from the aortic arch to the abdominal aorta, was significantly reduced in the darpladib group. Additionally, the expression of inflammatory genes monocyte chemotactic protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) was significantly reduced at the lesions in the darapladib group.

Conclusion: Inhibition of lp-PLA2 by darapladib decreases the inflammatory burden and atherosclerotic plaque formation in LDLR-deficient mice, which may be a new strategy for the treatment of atherosclerosis.

Keywords: atherosclerosis; lp-PLA2; darapladib; LDLR-deficient mice; inflammation; high-sensitivity C-reactive protein; interleukin-6; monocyte chemotactic protein-1; vascular cell adhesion molecule-1

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Introduction

Phospholipases A2 (PLA2s) constitute a superfamily of enzymes that catalyze the hydrolysis of fatty acids from the sn-2 position of glycerophospholipids to produce free fatty acids and lysophospholipids, which are both involved in signaling transduction and metabolic processes, and account for a vast number of diseases[1, 2]. PLA2s, then, are obvious candidates for pharmacological research and intervention. Of all the PLA2s, lp-PLA2 in particular has gained increasing attention as a growing number of epidemiological and experimental studies suggest that it plays an important role in diseases such as atherosclerosis, diabetes, and asthma[3–6].

Lp-PLA2, also referred to as platelet-activating factor acetylhydrolase (PAF-AH; E.C. 3.1.1.47), is a Ca^{2+}-independent, 45 kDa secreted protein that associates with lipoproteins and circulates within the plasma in active form[7]. Lp-PLA2 can be up-regulated by the oxidized phospholipids in oxLDL[8] and in turn acts upon those oxidized phospholipids to produce two pro-inflammatory mediators, lysophosphatidylcholines (lysoPCs) and oxidized nonesterified fatty acids (oxNEFAs)[9]. Research suggests that the regulatory roles of these two products, especially lysoPCs, are in the promotion of atherosclerotic plaque formation. For instance, lysoPCs have the capacity to recruit leukocytes to lesions, activate leukocytes to initiate immune responses, and promote foam cell formation[10–12].

The role of lp-PLA2 in the processing of oxLDL, inflammation, and atherogenesis suggests that the inhibition of this

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enzyme could play a positive role in the treatment of cardiovascular events. Indeed, earlier studies have reported that a specific lp-PLA2 inhibitor, darapladib, attenuates the inflammatory burden in patients with stable coronary artery disease and prevents necrotic core expansion, a key determinant of plaque rupture[13, 14]. In animal assays, the inhibition of lp-PLA2 by darapladib reduced complex coronary atherosclerotic plaque formation in pigs with induced diabetes and hypercholesterolemia[15].

However, there has been no *in vivo* data about the effects of lp-PLA2 inhibitor on the development of atherosclerosis in mouse models. In our study, we evaluated the specific lp-PLA2 inhibitor, darapladib, in the low density lipoprotein receptor (LDLR)-deficient mice to further confirm its role in the development of atherosclerosis.

Materials and methods

Chemicals

Darapladib was synthesized and procured from Prof Jian-hua SHEN's lab at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China. TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). PCR-related reagents were purchased from BIO-RAD (Hercules, CA, USA). Other reagents, unless otherwise specified, were obtained from Sigma-Aldrich (St Louis, MO, USA).

Animals

Male homozygous LDLR-deficient mice (C57/Bl6 genetic background) were obtained from the Jackson Laboratory. The animals were cared for in accordance with the institutional guidelines of the Animal Care and Use Committee of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

The mice were fed a high-fat diet consisting of 18% hydrogenated cocoa butter, 0.15% cholesterol, 7% casein, 7% sucrose, and 3% maltodextrin for 17 weeks, beginning at 6 weeks of age. Forty mice were divided into two groups (n=20 per group) randomly. One group received darapladib by gavage (50 $mg \cdot kg^{-1} \cdot d^{-1}$) once daily, while the other group received the vehicle (saline). During the 6 weeks of treatment, all mice were housed in a room with a 12-h light/dark cycle and were allowed free access to a high-fat diet and water.

Serum lipid analysis

Blood samples were obtained from the retro-orbital plexus of the mice prior to drug administration, and 24 h after the last round of drug administration. Serum was obtained through the centrifugation of blood at 1000×g and stored at -80 °C until analysis. Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglyceride (TG) levels were measured with an auto-analyzer (Hitachi 7100, Japan) using the corresponding kits from Wako Inc (Enid, OK, USA).

Measurement of serum Lp-PLA2 activity

Serum lp-PLA2 activity was measured using 2-thio-PAF as the substrate. Briefly, 10 μL of plasma was added to 0.1 mol/L Tris-HCl (pH 7.2) containing 1 mmol/L EGTA, 50 μmol/L 2-thio-PAF and 10 μL of 2 mmol/L 5,5′-dithiobis (2-nitrobenzoic acid) in a total volume of 200 μL. The assay was performed using a plate reader to obtain absorbance values at 414 nm every minute. The lp-PLA2 activity was calculated from the change in absorbance per minute.

Measurement of serum interleukin-6 (IL-6), high-sensitivity C-reactive protein (hs-CRP), and platelet activating factor (PAF)

Serum IL-6, hs-CRP, and PAF levels were determined by corresponding ELISA kits (Mingrui Biotech Inc, Shanghai, China).

Morphology of atherosclerotic plaques

At the end of the 6-week treatment period, the mice were anesthetized and killed. Several mice were perfusion-fixed with 4.5% formaldehyde. The aortas were dissected, from the heart to approximately 3 mm distal to the iliac bifurcation. The aortas were then preserved in fresh paraformaldehyde solution for 2 weeks and Sudan IV staining was employed to analyze plaque formation along the entire length of the aortas. Briefly, the aortas were opened longitudinally and pinned upon on a black silica gel plate after removing the surrounding adventitial fatty tissue. The aorta was rinsed in 70% ethanol after 12 h of fixation in the paraformaldehyde solution, stained with 1% Sudan IV in 50% acetone/35% ethanol for approximately 10 min, and washed in 80% ethanol for 5 min. Finally, the stained aortas were photographed and analyzed using the Image Pro-Plus 6.0 software.

Quantitative RT-PCR

After 6 weeks of treatment, the total RNA was extracted from the aortic arch and the thoracic-abdominal aortas of the two groups of mice using TRIzol reagent (Invitrogen Inc, Carlsbad, CA, USA). First-strand cDNAs were synthesized from 4 μg of total RNA using M-MLV reverse transcriptase (Promega Inc). Quantitative real-time PCR was performed using SYBR Green I as the detector dye. Relative gene expression was calculated by normalizing to the amount of mouse actin gene. The primer sequences are shown in Table 1.

Statistical analysis

Data are presented as mean values±SD. For serum lipid and lp-PLA2 activity, comparisons were made using the one-way analysis of variance (ANOVA), followed by the *post-hoc* Dunnett test for significance. Comparisons of body weight, plaque area and gene expression were analyzed by a two-tailed Student's t-test. For all tests, $P<0.05$ was considered statistically significant.

Results

Darapladib inhibits serum lp-PLA2 activity *in vivo*

Our previous unpublished research has shown that mouse lp-PLA2 is less sensitive to darapladib than human lp-PLA2. In this study, then, we tested the dosage range of darapladib to establish the dose at which lp-PLA2 in mouse serum would be

Table 1. Primer sequences for quantitative real-time PCR.

Gene	Forward (5'–3')	Reverse (5'–3')	Genebank ID
Lp-PLA2	GAGCGTCTTCGTGCGTTTG	GCGGGTATTTTTCTCCAGTC	NM_013737
MCP-1	CCTGCTGTTCACAGTTGCC	TGTCTGGACCCATTCCTTCT	NM_011333
ICAM-1	GCTGTATGGTCCTCGGCTG	GCCCACAATGACCAGCAGTA	NM_010494
VCAM-1	TGAACCCAAACAGAGGCAGA	CGGAATCGTCCCTTTTTGTAG	NM_011693
β-Actin	GGGAAATCGTGCGTGACA	CAAGAAGGAAGGCTGGAAAA	NM_007393

MCP-1, monocyte chemotactic protein-1; ICAM-1, Inter-Cellular Adhesion Molecule 1; VCAM-1, vascular cell adhesion molecule-1.

significantly inhibited. As shown in Figure 1, the activity of serum lp-PLA2 was inhibited by more than 60% in LDLR-deficient mice after oral administration of 50 mg/kg once daily of darapladib for 6 weeks.

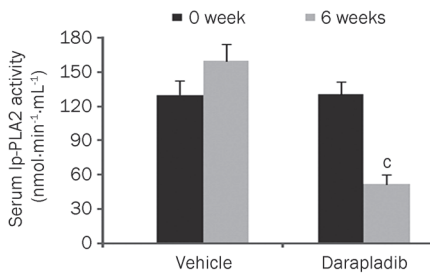

Figure 1. Darapladib significantly inhibits serum lp-PLA2 activity in LDLR-deficient mice. Serum lp-PLA2 activity was measured using spectrometry before and at the end of drug administration. $^{c}P<0.01$ *vs* vehicle at 6 weeks.

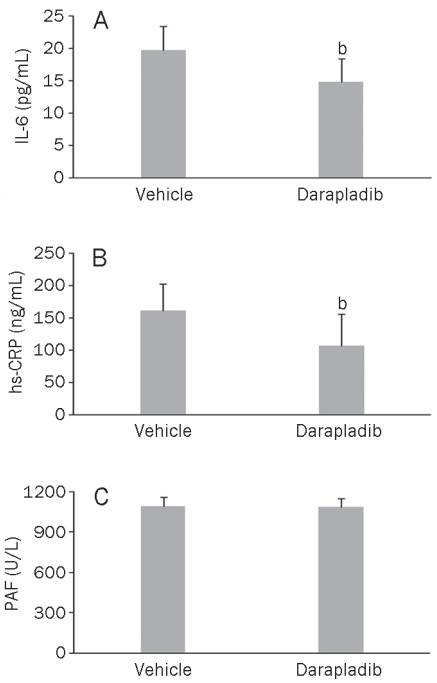

Figure 2. Inhibition of lp-PLA2 by darapladib decreases serum hs-CRP and IL-6 levels, but has no significant effects on the PAF level. After 6 weeks of treatment, serum hs-CRP, IL-6 and PAF levels were determined using ELISA. $^{b}P<0.05$ *vs* vehicle.

Inhibition of lp-PLA2 by darapladib has no significant effects on serum lipid profile

Lipid level and body weight were evaluated in both groups. As expected, there was no significant difference in TC, TG, LDL-C, and HDL-C levels between the two groups (Table 2). Additionally, there was no significant difference in the body weight of LDLR-deficient mice in either group (Table 2).

Inhibition of lp-PLA2 attenuates the inflammatory burden in serum

To evaluate the effects of lp-PLA2 inhibition on the inflammatory burden *in vivo*, we examined two typical inflammatory markers in the serum of LDLR-deficient mice by ELISA — hs-CRP and IL-6. As shown in Figure 2A and 2B, both hs-CRP and IL-6 were significantly reduced in the darapladib group as compared with those in the vehicle group. To determine whether inhibition of lp-PLA2 affects levels of PAF, we also examined the serum PAF. As shown in Figure 2C, plasma PAF levels between the two groups were similar.

Table 2. Effects of inhibition of lp-PLA2 by darapladib on body weight (g), serum total cholesterol, triglyceride, HDL-C and LDL-C levels (mmol/L) in LDLR-deficient mice.

	Body weight	Total cholesterol	Triglyceride	HDL cholesterol	LDL cholesterol
Vehicle	41.4±4.6	34.70±9.24	4.23±1.20	7.04±1.22	30.98±5.78
Darapladib	39.5±4.7	35.76±7.55	4.36±0.48	6.92±0.47	32.00±3.44

Inhibition of lp-PLA2 decreases the formation of atherosclerotic lesions

To determine the effects of the lp-PLA2 inhibitor on the development of atherosclerotic lesions in the aortic vessels of both groups of mice, we analyzed the plaque size at the proximal aorta by Sudan IV staining. As shown in Figure 3, the plaque content in the darapladib group, expressed as the occupancy of aortic luminal surface by atherosclerotic lesions, had significantly decreased compared with the plaque content in the vehicle group.

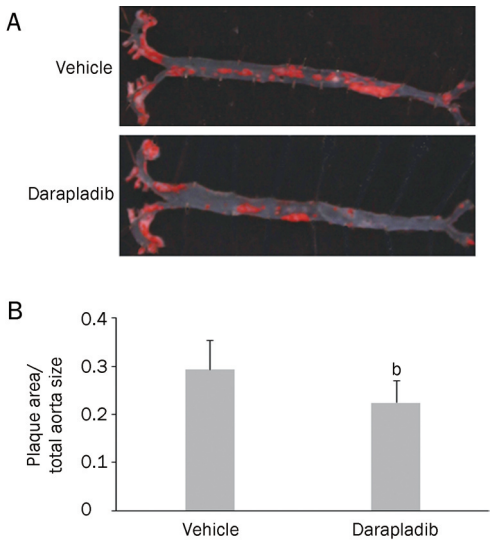

Figure 3. Inhibition of lp-PLA2 decreases the atherosclerotic area. (A) Representative en face atherosclerotic aorta preparations stained with Sudan IV. (B) Comparison of plaque sizes between the vehicle and darapladib groups (n=8 per group). ^{b}P<0.05 *vs* vehicle.

Inhibition of lp-PLA2 attenuates some inflammatory gene expression at plaque lesions

We additionally examined lp-PLA2, as well as the expression of several inflammatory genes in the aortic vessels, by quantitative real-time PCR. Interestingly, there was no significant difference in the expression of lp-PLA2 between the two groups (Figure 4). However, the levels of expression from the MCP-1 and the VCAM-1 genes were remarkably reduced in the darapladib group as compared to the vehicle group (Figure 4). Surprisingly, the expression of another inflammatory gene that we analyzed, ICAM-1, was comparable in the two groups (Figure 4).

Discussion

Atherosclerosis is the most common cause of many cardiovascular diseases, such as myocardial infarction and stroke[16]. It is widely recognized that the development of atherosclerosis is associated with both the metabolism of lipids and inflammation[17]. In our study, we examined the effects of a specific lp-PLA2 inhibitor, darapladib, on inflammation and atherogenesis in well-established LDLR-deficient mice. We found that the inhibition of lp-PLA2 does not change lipid profiles. Instead, the inhibition of lp-PLA2 attenuates the *in vivo* inflammatory burden and decreases atherosclerotic plaque formation in LDLR-deficient mice.

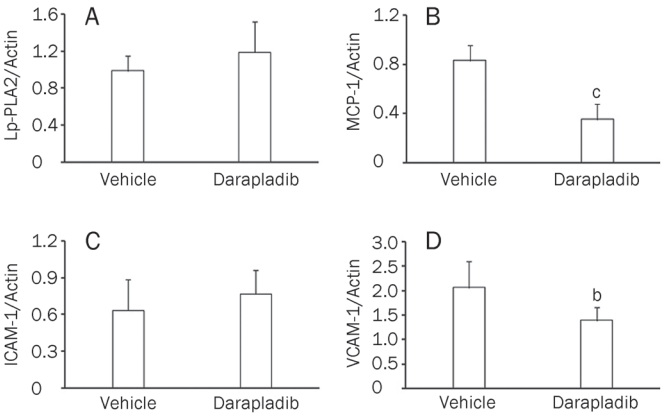

Figure 4. Inhibition of lp-PLA2 attenuates some inflammatory gene expression at the aortic arch and thoracic-abdominal aortas. Lp-PLA2, MCP-1, ICAM-1, and VCAM-1 gene expressions were determined using quantitative RT-PCR. n=5 per group. ^{b}P<0.05, ^{c}P<0.01 *vs* vehicle.

Specific gene-knockout mouse models are extensively used to study the pathological processes and pharmacological interventions of atherosclerosis, among which the model of the LDLR-deficient mouse is well established[18]. As the uptake of lipoprotein particles is impaired, the blood lipid profiles of these mice spontaneously rise. The high-fat diet leads to increased cholesterol, as well as atherosclerotic plaque formation[19]. As predicted, however, the treatment of these mice with the lp-PLA2 inhibitor did not alter their plasma lipoprotein profiles. The beneficial effects of the lp-PLA2 inhibitor, then, are not dependent upon the alteration of lipoprotein levels. The inhibition of lp-PLA2 has shown no marked influence on plasma lipoprotein profiles in pigs with induced diabetes and hypercholesterolemia (DM-HC) or in cardiovascular patients[13, 15, 20]. These findings are consistent with the results from our study. Interestingly, the elevation of *in vivo* lp-PLA2 activity by adenovirus-mediated gene transfer also does not alter the plasma lipoprotein profile[21].

Many epidemiological studies have suggested that lp-PLA2 is a marker for cardiovascular risk, and that the plasma levels and activity of lp-PLA2 increase during the development of atherosclerosis[22–30]. In our study, we also found that serum lp-PLA2 activity increased in the vehicle group after the mice were fed a high-fat diet for 6 weeks; such activity was significantly inhibited in the darapladib group.

Lp-PLA2 is thought to play an important regulatory role in the development of atherosclerosis due to the role of its enzyme activity in hydrolyzing bioactive lipids, such as PAF and oxidized phosphocholines (oxPCs). However, what researchers believe concerning the precise role of lp-PLA2 remains controversial. Lp-PLA2 may hydrolyze and inactivate

PAF, a well-known and typical pro-inflammatory factor that contributes to tissue damage and thrombosis formation[31]. On the other hand, lp-PLA2 may generate pro-inflammatory lipid mediators, such as oxNEFAs and lysoPCs, by hydrolyzing oxidized phospholipids in oxLDL. However, there is no evidence that lp-PLA2 hydrolyzes PAF *in vivo*. In our study, the inhibition of lp-PLA2 by darapladib did not change serum PAF levels. In addition, intravenous administration of recombinant lp-PLA2 failed to alter the PAF-mediated responses in patients with asthma or septic shock[32, 33]. Furthermore, a recent report suggested that circulating PAF is primarily cleared by transport and not by the hydrolysis of lp-PLA2 *in vivo*[34].

Conversely, there has been much *in vitro* or *in vivo* research that supports the concept of the pro-atherogenic roles of lp-PLA2. By hydrolyzing the oxidized phospholipids in oxLDL, lp-PLA2 produces two kinds of inflammatory mediators, lysoPCs and oxNEFA. These mediators initiate a high level of inflammatory response, such as cell adhesion, inflammatory gene expression, and cell death[10, 35]. In addition, *in vivo* studies have suggested that the inhibition of lp-PLA2 by darapladib decreases the inflammatory burden in humans and pigs[13–15]. In our study, we also examined the inflammatory factors in the serum and in the gene expression of some inflammatory cytokines in atherosclerotic lesions. Consistent with other *in vivo* studies, our research shows that the inflammatory burden decreased in the darapladib group compared with that in the vehicle group. Interestingly, previous research, along with our study, has suggested that the inhibition of lp-PLA2 did not affect lp-PLA2 expression. Theoretically, the inhibition of lp-PLA2 may increase lp-PLA2 expression at the vessels as with the oxPCs, which can up-regulate lp-PLA2 expression[8]. Thus, the expression of lp-PLA2 at atherosclerotic plaques needs further study. MCP-1, VCAM-1, and ICAM-1 are typical inflammatory cytokines mediating cell adhesion, a crucial step for monocyte migration into lesions[7]. LysoPCs, the product of lp-PAL2, can up-regulate MCP-1 and ICAM-1/VCAM-1 expression in the endothelial cells or vascular smooth muscle cells (VSMCs)[36]. Surprisingly, in our study, we detected the reduction of MCP-1 and VCAM-1, but not ICAM-1, expression. Regardless, our research, and the previous research of others, supports the pro-atherogenic roles of lp-PLA2 *in vivo*.

Indeed, the lp-PLA2 inhibitor has been evaluated in induced diabetes and hypercholesterolemia (DM-HC) in pigs[15]. Treatment with darapladib resulted in a considerable decrease in plaque area and a markedly reduced necrotic core area. Clinical research has also shown that the inhibition of lp-PLA2 with darapladib has arrested the expansion of the necrotic core, a key determinant of plaque vulnerability, despite not preventing plaque formation[13].

In summary, these *in vivo* studies demonstrate that the inhibition of lp-PLA2 by darapladib does not ameliorate dyslipidemia; instead, darapladib attenuates the inflammatory burden, resulting in the prevention of atherosclerosis in LDLR-deficient mice on a high-fat diet. Our study once again highlights that anti-inflammation therapy is a feasible strategy for the treatment of cardiovascular disease and lp-PLA2 is a promising target against atherosclerosis.

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Author contribution

Yi-ping WANG and Wen-yi WANG designed the research project; Yi-ping WANG supervised the project; Jie ZHANG, Miao-miao HU performed the animal assays; Wen-yu WU, Yan-ling MA, and Wei-hai CHEN performed the *in vitro* assays; Jie ZHANG, Miao-miao HU, and Wen-yi WANG analyzed the data and wrote the manuscript; Jie ZHANG, Miao-miao HU, and Yi-ping WANG revised the manuscript.

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