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Research Article

Inhibition of Adhesion Molecule Expression in Early Progression of Atherosclerosis in Dyslipidemia Model on Sprague Dawley Rats by Darapladib

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Abstract

Background and Objective: Dyslipidemia has been established as major risk factor contribute to atherosclerosis. One of emerging theory contribute to atherosclerosis is involvement of lipoprotein-associated phospholipase A2 (Lp-PLA2). Darapladib as inhibitor Lp-PLA2 have pro and contra about it is effect in atherosclerosis inhibition. This study aimed to examine the effects of darapladib on Lp-PLA2, intracellular adhesion molecule-1(ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in aortic tissue *in vivo*. **Materials and Methods:** This was a true experimental laboratory, *in vivo* post test only control group design, with 24 *Rattus norvegicus* Sprague Dawley strain rats were divided into six groups based on two serial time (normal group 8 weeks, dyslipidemic group 8 weeks, dyslipidemic group treated with darapladib 20 mg/200 g b.wt., 8 weeks and normal group 16 weeks, dyslipidemic group 16 weeks, dyslipidemic group treated with darapladib 20 mg/200 g b.wt., 16 weeks). The parameters of this study were Lp-PLA2, ICAM-1 and VCAM-1 measured by immunofluorescence and observed with a confocal laser scanning microscope in aortic smooth muscle cell. One way analysis of variance test and Pearson's correlation coefficient showed Darapladib had a significant effect ($p < 0.05$) in decreasing Lp-PLA2, ICAM-1 and VCAM-1 expression in aortic tissue of hypercholesterol diet given Sprague Dawley rat. **Results:** Darapladib 20 mg/200 g b.wt., is proven to decrease Lp-PLA2, ICAM-1 and VCAM-1 expression in aortic tissue *in vivo*. **Conclusion:** These finding provides a comprehensive mechanism inhibition of early progression of atherosclerotic process through inhibition of adhesion molecules by darapladib.

Key words: Darapladib, Lp-PLA2, ICAM-1, VCAM-1, dyslipidemia

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ischemic heart disease and stroke recently have been known as the leading cause of death in the world^{1,2}. While stroke becomes 1 out of 20 causes of death in US every year³. Additionally, stroke also becomes the most leading cause for long term disability³. Cardiovascular disease (CVD) and stroke are diseases caused by several risk factor such as smoking, physical inactivity, obesity, hypertension, diabetes and dyslipidemia⁴.

Dyslipidemia established as major risk factor for CVD and stroke⁵. Dyslipidemia is lipid metabolic impairment condition caused by interaction between genetic and environment factor which increase accumulation of low-density lipoprotein (LDL). In endothelium, LDL undergoes modification becomes oxidized-LDL, as a result of macrophage and endothelial cells activities. Lipid that accumulate in endothelium becomes the basis for endothelial dysfunction mechanism lead to formation of atherosclerosis⁶.

Recently one of emerging theories beyond the formation of atherosclerosis that widely studied is involvement of lipoprotein-associated phospholipase A2 (Lp-PLA2)⁷⁻⁹. Lp-PLA2, also known as platelet-activating factor acetylhydrolase (PAFAH) is a 45.4 kDa protein belongs to phospholipase A2 superfamily, encoded by *PLA2G7* gene and composes of 441 amino acids^{6,7,10}. This enzyme is produced by macrophage, monocyte, T-lymphocytes and mast cell in inflammatory condition and act to serine/aspartate/histidine substrate. Lp-PLA2 divided into secreted Lp-PLA2 that found in circulating system and Lp-PLA2 within atherosclerotic plaque. Secreted Lp-PLA2 approximately binds 70% to LDL-C and hydrolyze LDL into lysophosphatidylcholine (Lyso-PC). While Lp-PLA2 in atherosclerotic plaque hydrolyze oxidized LDL (Ox-LDL) into Lyso-PC and oxidized non-esterified fatty acid (Ox-NEFA)⁹. Lyso-PC and Ox-NEFA then stimulate expression of adhesion molecules, upregulate inflammatory cytokine, enhance matrix metalloproteinase expression, amplify oxidation and expand necrotic lipid core and thinning fibrous cap¹¹.

Intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are immunoglobulin superfamily that play important roles in adhesion of leukocytes to vascular endothelium¹². Expression adhesion molecules which is stimulated by Lyso-PC and Ox-NEFA is initial step of atherosclerosis formation. This mechanism explains the selective recruitment of mononuclear cells to arterial intima during early atherosclerosis. Immuno pharmacological blockage of ICAM-1 and VCAM-1 has been shown to inhibit fatty streak developments¹³.

Darapladib is selective Lp-PLA2 inhibitor developed by GlaxoSmithKline. Although darapladib has been proven for its pharmacokinetic effect in various clinical studies, but darapladib is failed to reduce primary end point target from two last phase III clinical trials¹⁴⁻¹⁹. It made darapladib becomes an interesting drug to be explored. This study aimed to know effect of darapladib treatment towards adhesion molecule, ICAM-1 and VCAM-1 and lipid profile in Sprague Dawley rats model dyslipidemia in early progression of atherosclerosis.

MATERIALS AND METHODS

Study design: Twenty four Sprague Dawley strain rats, 6-8 weeks old, with 150-200 g b.wt., were obtained from Bogor Agricultural University, Indonesia. Rats were divided into six groups after acclimatization. The control group was fed a standard diet (N), positive control group was fed a high fat diet (HFD) as authors previous study²⁰ (DL) and group with both HFD and administration of darapladib 20 mg/200 g b.wt., (DLDP). Each group is divided into 2 groups based on time serial, 8 and 16 weeks group. This study was conducted at the Biomedical Laboratory and Central Laboratory of Biological Sciences, Brawijaya University from February, 2015 until August, 2015 after obtaining ethical clearance assessment by the Health Research Ethics Committee.

Lipid profile measurement

Serum lipid profiles: Total cholesterol, high density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) were measured to ensure dyslipidemia model using EnzyChrom™ Assay Kit (E2HL-100) from BioAssay Systems.

Lp-PLA2, ICAM-1 and VCAM-1 measurement: Thoracic aortas were taken and cleaned of the surrounding fat fixed with PHEM buffer (68 mM PIPES, 25 mM, HEPES, pH 6.9, 15 mM EGTA, 3 mM MgCl₂, 10% (v/v) dimethyl sulfoxide containing 3.7% formaldehyde and 0.05% glutaraldehyde) as method used in Wihastuti *et al.*²¹, embedded in paraffin and sectioned 5 μm thickness for immunofluorescence examination. Each parameter then processed by immunofluorescence with anti-rat antibody Lp-PLA2 using rhodamin secondary antibody, anti-rat antibody VCAM-1 using fluorescein isothiocyanate secondary antibody and anti-rat antibody ICAM-1 using rhodamin secondary antibody (BIOS Inc., Boston, MA, USA). Three sections at interval 10 sections from each aorta (per rats) were taken. Each section were randomly captured at least five high power fields. The luminescences

were observed with confocal laser scanning microscopy (Olympus Corporation, Tokyo, Japan) and were quantitatively analyzed by Olympus FluoView software (version 1.7A; Olympus Corporation).

Statistical analysis: This study used one way analysis of variance (ANOVA) test to determine the effect of darapladib on the reduction of Lp-PLA2, VCAM-1 and ICAM-1 in Sprague Dawley strain *Rattus norvegicus* rats with hypercholesterol administration. Analysis was continued with Duncan *post-hoc* test ($p < 0.05$) to detect the differences of parameters among treatment groups. SPSS software (version 20; IBM Corporation, Armonk, NY, USA) was used for data analysis. Data are presented as mean \pm standard deviation.

RESULTS

Qualitative result from this study (Fig. 1) is measured by Olympus FluoView Software and it is presented in Table 1.

Expression of Lp-PLA2 in aortic tissue: Lp-PLA2 expression in group which received normal diet was lower compared to high fat diet (Table 1). Administration of darapladib could reduce Lp-PLA2 expression significantly compared to positive control group. The effect of darapladib reduced Lp-PLA2 expression in aortic tissue was clearly defined in 8 and 16 weeks treatment. Darapladib given in 16 weeks group was able to lower Lp-PLA2 expression in aortic tissue significantly compared to 8 weeks administration of darapladib.

Expression of VCAM-1 in aortic tissue: VCAM-1 expression in normal group were lower compared to high fat diet group. Darapladib was not able lowering VCAM-1 expression of aortic tissue in 8 weeks positive control group given darapladib. In 16 weeks, high fat diet group given darapladib, VCAM-1 expression reduced significantly compared to positive control group. However, VCAM-1 expression in 16 weeks high fat diet given darapladib

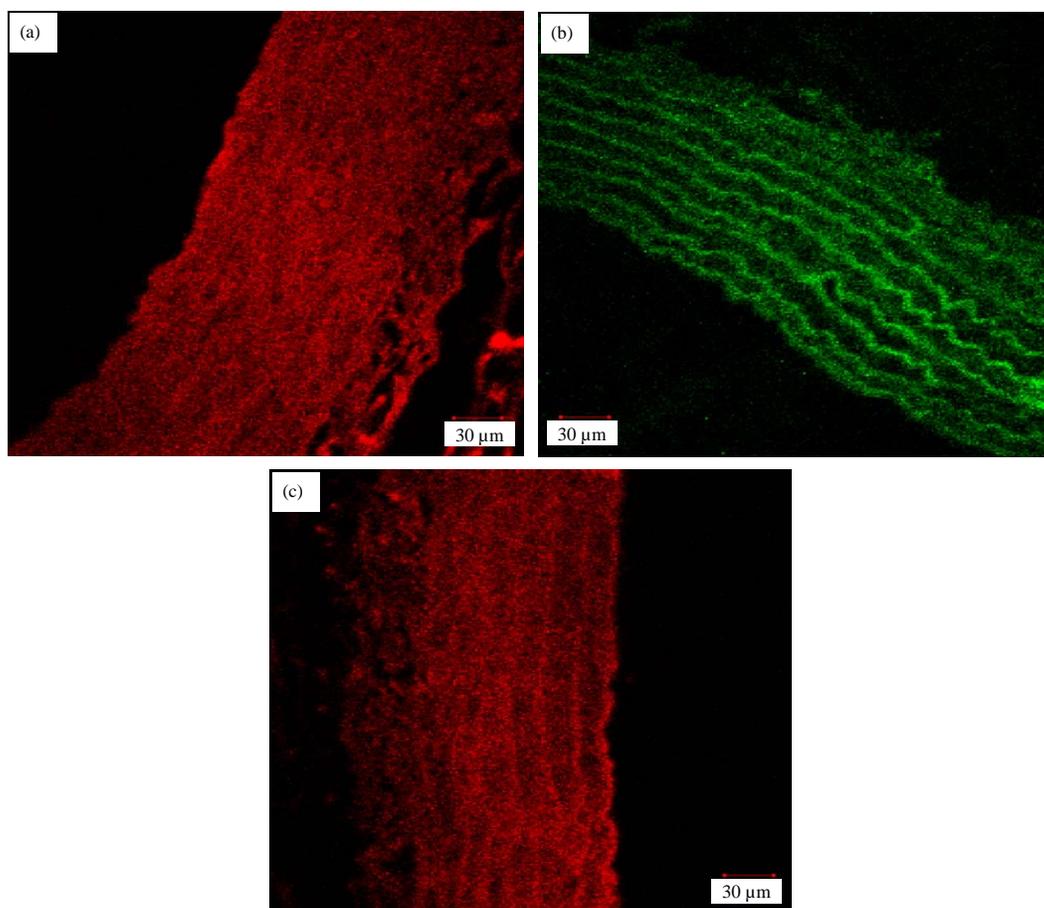


Fig. 1(a-c): Qualitative observation of Lp-PLA2, VCAM-1 and ICAM-1 expression on aortic tissue with immunofluorescence staining, (a) Lp-PLA2 with rhodamine, (b) VCAM-1 with fluorescein isothiocyanate and (c) ICAM-1 with rhodamine

Table 1: Parameter measurement, one-way ANOVA and *post-hoc* Duncan test for each group

Parameters	Lp-PLA2 (aU)	VCAM-1 (aU)	ICAM-1 (aU)
Normal 8 weeks	755.81 ± 4.19 ^a	562.34 ± 21.2 ^a	537.57 ± 17.38 ^{ab}
Dyslipidemia 8 weeks	1171.65 ± 52.82 ^d	738.12 ± 5.58 ^b	672.43 ± 8.62 ^b
Dyslipidemia+darapladib 8 weeks	977.23 ± 27.24 ^c	653.77 ± 25.32 ^{ab}	487.14 ± 34.14 ^a
Normal 16 weeks	677.23 ± 37.04 ^{ab}	649.92 ± 86.13 ^{ab}	400.07 ± 91.61 ^a
Dyslipidemia 16 weeks	899.16 ± 80.12 ^c	1169.47 ± 166.39 ^d	920.39 ± 240.17 ^c
Dyslipidemia+darapladib 16 weeks	648.47 ± 68.76 ^a	925.62 ± 95.88 ^c	448.93 ± 28.88 ^a
p-value	0.00	0.00	0.00

Data are presented as mean ± standard deviation (range) values. All the values of the parameters have been corrected into International Standard of Mathematics (decimals). Different letters indicate a statistically significant difference (Duncan *post-hoc* test $p < 0.05$), ANOVA: Analysis of variance, HDL: High density lipoprotein, LDL: Low density lipoprotein, VLDL: Very low density lipoprotein, Lp-PLA2: Lipoprotein-associated phospholipase, ICAM-1: Intracellular cell adhesion molecule-1, VCAM-1: Vascular cell adhesion molecule-1, aU: Arbitrary unit. Lipid profile can be seen in researchers previous publication²³

group still higher compared to 8 weeks high fat diet given darapladib group significantly.

Expression of ICAM-1 in aortic tissue: ICAM-1 expression in normal group were lower compared to high fat diet group significantly in 16 weeks group but not in 8 weeks group. Darapladib administration could reduce ICAM-1 expression significantly in positive control group in 8 and 16 weeks group. Reduction of ICAM-1 expression in high fat diet given darapladib group in 8 weeks compared to 16 weeks were not significantly different.

Pearson correlation: Pearson correlation test showed a significant result and strong positive correlation between Lp-PLA2 with VCAM-1 expression and ICAM-1 expression both in 8 weeks ($r = 0.918$, $p = 0.000$ and $r = 0.650$, $p = 0.022$) and 16 weeks ($r = 0.841$, $p = 0.001$ and $r = 0.792$, $p = 0.002$) of atherosclerosis.

DISCUSSION

High fat diet induce abnormal amount of lipids in the blood or dyslipidemia which most of them are hyperlipidemia which characterized by elevation of total cholesterol and/or elevation of low-density lipoprotein (LDL) cholesterol and/or elevation of triglyceride concentrations and/or decrease of high-density lipoprotein (HDL) cholesterol in the blood²². Lipid profile result from researchers previous publication was in line with this theory²³.

Lipid normally stored in adipose tissue as an esterified lipid, but in high fat diet 40-50% lipid undergo spillover causes ectopic fat deposition²⁴. Fat deposition in vascular causes infiltration of apoB containing lipoproteins (LDL, remnant VLDL and chylomicrone) in the artery wall. The retained lipoproteins induce ROS production that lead to endothelial dysfunction. ROS then modifying LDL into Ox-LDL and Ox-LDL then induce more ROS formation²⁰. In line with this theory,

researchers previous publication showed that high fat diet proved induce Ox-LDL formation and darapladib administration could reduce Ox-LDL level in serum and aortic tissue²⁴.

Lp-PLA2 produced mainly by proinflammatory cells such as monocytes, macrophages, T-lymphocyte and mast cell. Secreted Lp-PLA2 approximately binds 70% to LDL in circulation and hydrolyze LDL into lysophosphatidylcholine (Lyso-PC). While Lp-PLA2 in atherosclerotic plaque hydrolyze Oxidized LDL (Ox-LDL) into Lyso-PC and oxidized non-esterified fatty acid (Ox-NEFA)²⁵. In various clinical study, Lp-PLA2 proved to be a significant marker of cardiovascular events^{26,27}. This study also found that Lp-PLA2 expression in aortic tissue was higher in high fat diet group compared to normal group harmonious with established theory. However, in 16 weeks high fat diet group have a lower Lp-PLA2 expression compared to 8 weeks high fat diet group. This though because Lp-PLA2 work mainly in circulation bind to LDL²⁸.

Oxidative stress has important role in pathophysiology of atherosclerosis. Sufficient levels of ROS, particularly superoxide anion and hydrogen peroxide has been shown to activate nuclear factor kappa beta (NF- κ B)²⁰. NF- κ B are transcription factors which bind to VCAM-1 and ICAM-1 promoter and induce VCAM-1 and ICAM-1 gene transcription. This process promote the binding, rolling and stable arrest of inflammatory white blood cells, such as T cells, monocytes and mast cells²⁹. Cytokine production by inflammatory cells within endothel like TNF- α and IL-1 also works on endothelial cell and induces endothelial cells to express VCAM-1 and ICAM-1 via NF- κ B activation. The ICAM-1 and VCAM-1 play a major role in the initiation of early atherosclerosis, mainly its contribution to monocyte adhesion. Inhibition of the inflammatory response is widely known to be beneficial in the early stages of atherosclerosis³⁰.

Result from present study shown a significant elevation of VCAM-1 expression in high fat diet group compared with

normal group in line with the theory. VCAM-1 expression significantly higher in 16 weeks high fat diet group compared to 8 weeks group shows that VCAM-1 more likely have a role in long term atherosclerosis process. Clinical study of VCAM-1 expression also shown that VCAM-1 expression is higher in patients with chronic already-established lesion such as in coronary artery disease and have a positive correlation with plaques extension. Moreover, VCAM-1 also become one of predictor biomarker of future cardiovascular events in patients with coronary artery disease, diabetes mellitus and unstable angina³¹. Darapladib reduction of VCAM-1 expression in 16 weeks high fat diet group still far from normal VCAM-1 level in 16 weeks normal group. Thus, showed that darapladib administration eventhough can reduce VCAM-1 expression in high fat diet, it couldn't lowering VCAM-1 expression reach out the normal level. This result in harmonious with the clinical phase III trial of darapladib, SOLID-TIMI and STABILITY study which shown that darapladib did not significantly reduce the primary end point cardiovascular death, but only reduced the rate of the secondary end point of major coronary events²⁸. In present study, darapladib could also decreasing ICAM-1 expression in 8 and 16 weeks eventhough ICAM-1 expression lowered by darapladib was not different significantly between 8 weeks administration and 16 weeks administration.

In early progression of atherosclerosis, ICAM-1 and VCAM-1 has an important role to initiate formation of atherosclerotic plaque by facilitate monocyte/macrophage infiltration. As soon as the macrophage infiltrates of tunica intima, it will differentiates into macrophage that ingest Ox-LDL and develops into foam cells. Reduction of VCAM-1 and ICAM-1 expression by darapladib administration in this study proved that darapladib could reduce immune cell (monocyte etc) infiltration, inhibit the inflammatory process and finally inhibit atherogenesis³². In harmonious with the result in this study, effect of darapladib in decreasing foam cell has been prove in our previous study²⁴.

This study showed that reduction of Lp-PLA2 have a beneficial effect on lowering ICAM-1 and VCAM-1 consistent with phase II trial of darapladib. However darapladib administration in the last phase III trial STABILITY and SOLID TIMI study seems failed to achieve the primary end point of cardiovascular death. The accumulated studies show that although Lp-PLA2 activity was associated with progression of atherosclerosis and increased of cardiovascular events, but lowering Lp-PLA2 activity was not useful to prevent cardiovascular events in human due to many reasons.

CONCLUSION

Darapladib 20 mg/200 g b.wt., is proven to decrease Lp-PLA2, ICAM-1 and VCAM-1 expression in aortic tissue *in vivo*. These finding provides a comprehensive mechanism of inhibition of early progression of atherosclerotic process through inhibition of adhesion molecules by darapladib.

SIGNIFICANCE STATEMENT

This study discovers a significant effect of darapladib on lowering Lp-PLA2, ICAM-1 and VCAM-1 that can be beneficial for early progress of atherosclerosis in dyslipidemia model Sprague Dawley rats. The unsatisfactory effect of darapladib on lowering ICAM-1 and VCAM-1 in 16 weeks model could uncover reasons beyond darapladib's failure to achieve the primary endpoint in clinical trial phase III that many researchers were not able to explore.

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