The effect of darapladib administration to inflammation marker in early development of atherosclerosis: in vivo study for dyslipidemia model

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Abstract. Dyslipidemia is a chronic inflammation condition which related to Lp-PLA2. LpPLA2 has an anti-inflammatory role as it hydrolyze atherogenesis mediators. These mediators, such as oxLDL to produces lyosphophatidylcholine (lysoPC) and oxidized fatty acid (oxFA) that have pro-inflammatory, proliferative and pro-atherogenic effect. This study aimed to discover the expression of inflammation marker of dyslipidemia in vivo model with darapladib treatment. A true experimental laboratory and only posttest with control group design used 30 Spraque Dawley rats which were divided into three main groups: normal, dyslipidemia, and dyslipidemia with darapladib administration. Each group consisted of two serials treatment time: 8-weeks and 16-weeks. Parameter measured was ox-LDL level, TNF-α, iNOS, IL-6, PAF and PAT thickness and also lipid profile. The study results analyzed using ANOVA test showed that darapladib were significantly (p <0.05) lowering expression of Ox-LDL in aortic tissue, blood Ox-LDL and IL-6 in vivo model of dyslipidemia. This study concludes that dalapladib proved to have role to decrease Ox-LDL in aortic tissue, blood Ox-LDL and IL-6 significantly in vivo model of dyslipidemia.

1. Introduction

Major cause of death in developed and developing countries is cardio vascular disease (CVD). The death rate from CVD in Indonesia reaches account for 37% of total deaths. CVD was the second leading cause of death in 2012 representing 9% of deaths (138,400 people) in Indonesia.[1]. In the world, there are 17.5 million people that die each year because of CVD and 80% of all CVD deaths are due to heart attacks and strokes [2]. One of the common cause of CVD is atherosclerosis, including coronary heart disease (coronary artery disease), cerebrovascular disease (stroke) and peripheral blood vessel diseases (peripheral artery disease)[3].

One of the main classic risk factors for atherosclerosis and CVD is dyslipidemia [4]. Enzymatic activity and expression of lipoprotein associated phospholipase A2 (Lp-PLA2) have begun to be studied as a biomarker of CVD risk in people [5]. The understanding about role of Lp-PLA2 in the pathophysiology of cardiovascular disease remains controversial and far from well established. Lp-
PLA2 has antiinflammatory role by the ability to hydrolyze mediators in atherogenesis such as ox-LDL and platelet activating factor (PAF) and also the hydrolysis products of these molecules such as lysophosphatidylcholine (lysoPC) and oxidized fatty acid (oxFA) are of pro-inflammatory. Currently, there are many researches that look for novel treatment to decrease occurrence of atherosclerotic cardiovascular disease [6]. Atherosclerotic lesion is characterized by highly expression of lipoprotein associated phospholipase A2. Many studies shown Darapladib can reduce activity of LpPLA2 in human carotid plaque. Darapladib becomes a potent and reversible inhibitor of LpPLA2 [7]. Darapladib can inhibit atherosclerosis significantly in several studies in vitro and in vivo experiment [7, 8, 9]. However, there are several opinions that show negative results of darapladib in reducing mortality from cardiovascular disease, myocardial infarction, or stroke [10]. The SOLID-TIMI 52 Randomized Clinical Trial showed that patients who experienced an ACS event, direct inhibition of LpPLA2 with Darapladib did not decrease the risk of major coronary events [10]. Based on those facts, this study aimed to determine the expression inflammation marker of dyslipidemia in vivo model with darapladib treatment.

2. Material and Methods

2.1 Study Design

We used male Sprague Dawley rat species aged 4 weeks old with the body weight around 150-200 gram were obtained from Bogor Agricultural University (IPB), Indonesia. The rats were divided into three different groups; including control/normal group (N); Dyslipidemia groups (DL) given high fat diet (HFD), and dyslipidemia with Darapladib treatment group (DLDP). Each group was divided into two serial times which were 8 and 16 weeks. Darapladib was obtained from Glaxo Smith Kline from United States. The rats were given Darapladib orally 20 mg/Kg body weight once daily in duration according the time serial groups given. N8 and N16 for normal groups with 8 and 16 weeks. DL8 and DL16 for DL model groups with 8 and 16 weeks and DLDP8 and DLDP16 for dyslipidemia model with Darapladib treatment groups in 8 and 16 weeks. The number of control and experimental group is described in Table 1 below.

| Group   | Number of Sample | Diet and Treatment Period          |
|---------|------------------|-----------------------------------|
| N 8     | 5                | Normal Diet in 8 weeks            |
| N 16    | 5                | Normal Diet in 16 weeks           |
| DL 8    | 5                | HFD in 8 weeks                    |
| DL 16   | 5                | HFD in 16 weeks                   |
| DLDP 8  | 5                | HFD + DP 20 mg/kg/day in 8 weeks  |
| D DP 16 | 5                | HFD + DP 20 mg/kg/day in 16 weeks |

Preparation phase is aimed to adapt the rat with new environment for two weeks. During this phase, all of rats were given Normal Diet. Early of third week, 10 rats were given Normal Diet while 20 rats were chosen randomly for making DL rat model. Treatment phase was started in week eight when the rats age 13 - 15 weeks. In this phase the rats are fed according to the research plan as described above. Particularly in the treatment group administered Darapladib 20 mg / kg body weight per oral / day during the treatment period. At the end of treatment phase, euthanasia was done by injecting ketamine 15-20 mg / kg per intra peritoneal. Anesthesia and surgery were performed by researchers and laboratory staff. Blood was taken from the heart, then placed in venojec. Blood plasma samples were centrifuged 3000 rpm for 10 minutes. It is stored immediately at -80 ° C. Plasma will be
used for examination of lipid profiles (total cholesterol, HDL, LDL / VLDL and non-HDL cholesterol) and PAF.

The aortic tissue is cut and substituted in eppendorf for calculation of TNF-α, IL6 and iNOS cytokines. The aorta was washed with PBS to remove residual blood clot. Then taken 1/3 of aortic tissue and stored it at -20 °C. Then it will be stored in paraffin block, using xylol, 96% alcohol, paraffin, Formalin / PFA.

2.2 Biochemical tests
2.2.1 IL-6, TNF-α and iNOS expression
We used immunofluorescence for measuring IL-6, TNF and iNOS of aortic tissues that were previously fixed with PHEMO buffer (68 mM PIPES, 25 mM HEPES, pH 6.9, 15 nMEGTA, 3 mM MgCl2, 10% [v/v] dimethyl sulfoxide containing3.7% formaldehyde and 0.05% glutaraldehyde) and were processed by immunofluorescence labeling with anti-rat antibody IL-6 using rhodamin secondary antibody and anti-rat antibody TNF-α and iNOS used fluorescein isothiocyanate (FITC) secondary antibody (BIOS Inc., Boston, MA, USA). We observed them with confocal laser scanning microscopy (OlympusCorporation, Tokyo, Japan) and were analyzed using Olympus FluoView software (version 1.7A; Olympus Corporation) quantitatively.

2.2.2 OxLDL and PAF measurement
Ox-LDL was measured using rat’s aorta tissue as samples. Rats had been fasted a day before the sample obtained. Measurement of Ox-LDL level and PAF concentrations in blood plasma samples of rat conducted by ELISA method. Ox-LDL level was measured with Rat ox-LDL ELISA Kit (Cat. No. E-EL-R0710) and PAF concentration was measured with Rat Platelet Activating Factor (PAF) ELISA kit (Cat. No. MBS722041). Competitive ELISA begins with Coating antigen. Standard and sample of 100 μL were inserted into the well. The sample was (except blank well) was incubated at 37°C for 1 hour. Then we added 50 μL Substrate A and 50 μL Substrate B to each well and incubated for 10-15 minutes at37°C (avoid from light). We stop the reaction by adding 50 μL stop solution to each well. After 5 minutes, we read them by ELISA reader at 450 nm.

2.3 Ethic statement
This study has been evaluated and approved by Indonesian Health Research Ethics Committee through approval No 400/EC/KEPK/10/2016.

2.4 Statistical analysis
One-way ANOVA test was used to determine the effect of darapladib administration to vasa vasorum quantity in two serial times, in 8th and 16th week. Furthermore, analysis was performed using Post Hoc Duncan test to understand the differences of each parameter in each group. This statistical test was conducted using SPSS 16 (IBM Cooperation, New York, NY).

3. Result and Discussion
3.1 Effect of Darapladib on OxLDL Levels in Aortic Tissue
OxLDL of aortic tissue in DLDP group was decreased significantly, both at 8th and 16th weeks (p<0.05). The levels of OxLDL at 16th weeks was higher than at 8th weeks. In the blood, OxLDL levels in DLDP group was decreased but there was no significant difference between 8th and 16th weeks of Darapladib administration.
Table 2. Lipid profile

| Variable          | Group  | 8th weeks mean± SD | 16th weeks mean± SD |
|-------------------|--------|-------------------|---------------------|
|                   |        |                   |                     |
| Total cholesterol | Normal | 72.80 ± 4.05 b     | 56.56 ± 5.43 a      |
|                   | DL     | 109.56 ± 12.25 cd  | 100.57 ± 22.64 c    |
|                   | DLDP   | 79.57 ± 10.54 b    | 96.96 ± 18.97 c     |
| HDL               | Normal | 34.74 ± 8.31 f     | 35.77 ± 1.68 f      |
|                   | DL     | 19.16 ± 0.30 ab    | 17.55 ± 0.85 de     |
|                   | DLDP   | 20.62 ± 0.20 c     | 13.00 ± 1.82 b      |
| Non-HDL           | Normal | 49.83 ± 5.06 b     | 19.24 ± 3.67 a      |
|                   | DL     | 88.20 ± 3.08 ab    | 98.14 ± 11.43 c     |
|                   | DLDP   | 60.34 ± 2.64 c     | 96.51 ± 17.06 de    |

Note: On different trials 2 means with duncan multiple range test (DMRT), there is a similar letter notation, which means the difference between two mean value not significant. *α = 0.05.

Table 3. Inflammation markers

| Variable      | Group  | 8 weeks Mean± SD | 16 weeks Mean± SD | P value |
|---------------|--------|------------------|-------------------|---------|
|               |        |                   |                   |         |
| Tissue Ox LDL | Normal | 1.35 ± 0.16 a     | 2.31 ± 0.27 ab    | 0.000   |
|               | DL     | 12.75 ± 0.66 f    | 15.78 ± 0.76 c    |         |
|               | DLDP   | 3.49 ± 0.12 bc    | 6.49 ± 0.78 a     |         |
| Blood oxLDL   | Normal | 0.22 ± 0.06 a     | 0.49 ± 0.36 bc    | 0.000   |
|               | DL     | 1.94 ± 0.11 c     | 2.02 ± 0.12 e     |         |
|               | DLDP   | 0.33 ± 0.06 ab    | 0.69 ± 0.08 d     |         |
| TNF-α         | Normal | 1283.12 ± 55.08   | 792.55 ± 91.29    | 0.094   |
|               | DL     | 1730.87 ± 200.8   | 676.57 ± 111.5    |         |
|               | DLDP   | 1424.18 ± 129.8   | 680.07 ± 28.75    |         |
| iNOS          | Normal | 555.65 ± 139.28   | 687.56 ± 217.82   | 0.064   |
|               | DL     | 516.58 ± 126.18   | 1264.04 ± 237.84  |         |
|               | DLDP   | 518.86 ± 63.35    | 923.02 ± 188.14   |         |
| IL-6          | Normal | 716.09 ± 42.19 ab | 670.56 ± 158.24 ab| 0.022   |
|               | DL     | 856.00 ± 352.02 ab| 947.00 ± 205.01 b |         |
|               | DLDP   | 727.23 ± 28.33 ab | 557.47 ± 244.45 a |         |
| PAF           | Normal | 0.79 ± 0.11 a     | 0.96 ± 0.12 ab    | 0.000   |
|               | DL     | 1.24 ± 0.12 cd    | 1.62 ± 0.19 e     |         |
|               | DLDP   | 1.06 ± 0.11 bc     | 1.27 ± 0.15 cd    |         |
| PAT           | Normal | 501.42 ± 67.90 ab | 478.90 ± 63.22 ab | 0.000   |
|               | DL     | 561.36 ± 74.38 bc | 519.39 ± 122.74 ab|         |
|               | DLDP   | 494.72 ± 142.24 abc| 388.25 ± 35.15 e  |         |

Note: On different trials 2 means with duncan multiple range test (DMRT), there is a similar letter notation, which means the difference between two mean value not significant. *α = 0.05.
3.2 Effect of Darapladib Administration on TNF-α Expression of Aortic Tissue

TNF-α of aortic tissue was decreased in DLDP group in 8 weeks group but there was no significant difference between the groups (p>0.05). TNF-α was not decreased after Darapladib administration in 16 weeks. The result was shown by Immunofluorescence staining using secondary FITC antibody (Figure 1.)

3.3 Darapladib Effect in iNOS Expression of Aorta Tissue

ANOVA test showed there was no significant difference of iNOS expression between each group (p>0.05). But iNOS was decreased in 16 weeks after darapladib administration. Immunofluorescence staining of iNOS using secondary antibody FITC was shown in Figure 1.

3.4 Darapladib Effect in IL-6 Expression of Aorta Tissue

In dyslipidemia group, Darapladib decreased IL-6 expression significantly (p<0.05) in 16 weeks. IL-6 expression was lower in 16 weeks compared to 8 weeks. Immunofluorescence staining of IL-6 expression using secondary antibody rhodamin was shown in Figure 1.

3.5 Darapladib Administration Effect to the Plasma PAF Level

PAF level was decreased in 8 and 16 weeks with Darapladib administration but it was not significantly (p>0.05). It was decreased higher in 16 weeks compared to 8 weeks.

3.6 Darapladib Administration Effect to the Perivascular Adipose Tissue Thickness

Darapladib can decrease PAT thickness in dyslipidemia group both in 8 or 16 weeks but not significant (p>0.05). PAT thickness in dyslipidemia group 16 weeks was more decreased compared to 8 weeks (Figure 2).

3.7 Darapladib Effect on iNOS, TNF-α and IL-6 Expression at Early Stage of Atherosclerosis

Atherosclerosis has been involved in inflammatory processes in the various stages of atherosclerosis. Smooth muscle and endothelial cells inside atherosclerotic plaque produce pro-inflammatory mediators that stimulate monocyte differentiation process into macrophage [11]. It is developed into foam cells and there is OxLDL uptake process [12]. Macrophages inside atherosclerotic plaques express iNOS that produce large amount of NO. Some of the adverse effects associated with iNOS
that produce excessive peroxinitrite and superoxide. In the other hand, iNOS also has protective role to inhibit smooth muscle cell proliferation and leukocyte adhesion [12].

![Figure 2](image_url)

**Figure 2.** PAT thickness in N8, N16, DL 8 and DL 16 mice group. Black arrow shows PAT thickness. Magnification 400x (A) Normal 8 weeks mice group (N8); (B) Normal 16 weeks mice group (N16); (C) 8 weeks dyslipidemia mice group (DL8); (D) 16 weeks dyslipidemia mice group (DL16) (E) Dyslipidemia mice group with Darapladib administration for 8 weeks (DLDP8). (F) Dyslipidemia mice group with DP administration for 16 weeks (DLDP16).

![Figure 3](image_url)

**Figure 3.** Tunica intima thickness of Sprague-dawley rat in each group

The result of this study showed that IL-6 expression in dyslipidemia group is higher than normal group. IL-6 expression was higher at 16 weeks compared with those 8 weeks. Administration of 30
mg/kg/day darapladib decreased the expression of IL-6 in the aortic tissue at dyslipidemia group significantly (p<0.05), mainly in 16 weeks group.

This research support previous study’s finding about increased level of inflammatory markers in pro-atherogenic conditions such as dyslipidemia which induced atherosclerotic lesions formation. Research about atherosclerotic plaques at human found significant association between increased levels of Lp-PLA2 and lysoPC in atherosclerotic plaques with increased expression of IL-6, IL-1β and TNF-α in atherosclerotic plaque [13]. Increased level of pro-inflammatory mediators (IL-6 and iNOS) was even more evident in 16 weeks observation group. The pattern of Lp-PLA2, IL-6 and iNOS expression in this study proves the theory about Lp-PLA2 in oxLDL that able to stimulate pro-inflammatory cytokines formation (especially IL-6, IL-1β and TNF-α) [15]. Strong association between IL-6 expression with Lp-PLA2 activity in this study can also be seen with decreased expression of IL-6 in aortic tissue with DP administration especially 16 weeks observational group. The result of this study are consistent with previous study, either using experimental animals or human tissue. The previous in vivo study reported a significant reduction in IL-6 levels post 50 mg/kg body weight/day DP administration in HFD mice for 6 weeks. The pattern of the same invention obtained by hs-CRP levels measurement the significantly depressed by the DP therapy [14].

Expression of iNOS in aorta rat did not have a significance value (p> 0.05) thus it could be concluded that administration of different treatment did not cause significant difference in iNOS expression. There was different pattern of TNF-α expression in aortic tissue at this study. Expression of TNF-α was increase only between DL 8 weeks and N 8 weeks. ANOVA test showed there is no difference between the group (p>0.05). Thus it could be concluded that administration of different treatment did not cause significant difference in iNOS expression.

3.8 Darapladib Administration Effect to the Plasma PAF Level
In this study, PAF plasma of mice decreased between DL group at 8-week and DLDP group at 8 weeks and also between DL 16 weeks and DLDP 16 weeks but not significantly. PAF role on the Lp-PLA2 was controversial. In a study conducted by Hu et al. darapladib did not alter the levels of serum PAF [16]. Another study revealed no changes in serum PAF levels by administering darapladib in an atherosclerosis mice model [17]. In this study, after darapladib administration, there was decrease in the level between DLDP group at 16 weeks and DL group at 16 weeks (p <0.05) and decrease between DLDP group at 8 weeks and DL group at 8 weeks although not significant (p> 0.05). PAF is formed as a result of acetylation of Lyso-PC. In general, PLA2 hydrolyzes phospholipid membrane, phosphatidylecholine (PC) and/or phosphatidylethanolamine (PE) to produce AA and lyso-PC or lyso-PE. Lyso-PC generated will bind with acetyl groups and form PAF [15] Therefore when Lp-PLA2 was inhibited then oxLDL hydrolysis would not occur. Subsequently there was no Lyso-PC formation. With the absent of lysoPC there would be no PAF formation through acetylation.

3.9 Effect of Darapladib Administration to Perivascular Adipose Tissue Thickness
Body with metabolic disorders or cardiovascular disease are generally accompanied by PAT. They cause PAT becomes hypoxic and dysfunctional as well as recruit phagocytic cells. Changes in cell size of adipocytes and an increase in macrophages and the infiltration of T cells decrease the production of adipokines which is protective and increase the production of pro-inflammatory adipokines such as leptin, resistin, kemerin, vaspin, IL-6, or TNF-α, both on human and mouse adipocytes tissue. Previous study report that the concentration of IL-6 and TNF-α was also higher in patients with CAD compared to healthy individuals with PAT [16].

Different pattern was found in this study about PAT thickness in the conditions of dyslipidemia. There was an increase in PAT thickness at 8- and 16-weeks group. But the extension of administration period does not cause PAT thickness becomes higher. Darapladib administration in dyslipidemia condition consistently decreased the thickness of PAT. Darapladib administration period was significantly reducing the PAT thickness. There was significant decrease in PAT thickness with Darapladib administration in 16 weeks administration compared with 8 weeks group.
Increased thickness PAT was more dominant in the treatment of dyslipidemia that was triggered by HFD administration and also consistent with the theory that suspect there was a significant relationship between the thickness of the PAT with a high intake of fat, obesity and resistance insulin [20]. The study of relationship between Lp-PLA2 activities with a thickness of PAT is very interesting because there has been no previous study report. Similar action of PAT in promoting local inflammatory response and constituted by direct contact that closely mimic the activity of Lp-PLA2 in triggering the inflammatory response. The research found Darapladib ability to suppress the thickness of the PAT in conditions of dyslipidemia (8 and 16 weeks) clearly demonstrating the association of both pro inflammatory agents. PAT trigger inflammatory responses and also strongly associated with macrophages. Macrophages have unique properties within PAT tissue. In proinflammatory condition such as dyslipidemia, obesity and T2DM, macrophages will increase pro-inflammatory cytokines and recruit more macrophage proinflammatory (M1) in PAT tissue. DP administration lead to a disconnection of Lp-PLA2 activity in oxLDL so that the number of macrophages in oxLDL and Lp-PLA2 which is formed as a result of the advanced stage will be also decreased [17].

4. Conclusion
Darapladib administration results in the decrease of Ox LDL level in the blood and aortic tissue, and IL-6 expression of Sprague Dawley rats with high fat diet.

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