The effects of specific inhibitor Lp-PLA2 on vasa vasorum angiogenesis through inhibition vascular endothelial growth factor expression: study in vivo using type 2 diabetes mellitus

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Abstract. Insulin resistance decreases endothelin’s ability to release NO, O₂, and other substances which causes inflammation and endothelial dysfunction. As a result, atherosclerotic plaques are easily formed. Hypoxia due to arterial wall thickening causes increase of vasa vasorum and HIF-1α secretion which in turn induce the production of VEGF causing angiogenesis. Darapladib is a Lp-PLA₂ inhibitor predicted to slow atherosclerosis progressivity. Experimental study used posttest with control group to Sprague Dawley, divided into 3 groups, 1) control groups, 2) type 2 diabetes mellitus group with high fat diet and 3) type 2 diabetes mellitus with high fat diet and darapladib administration. Measured parameter were glucose level, plasma insulin level, insulin resistency, lipid profile, amount of vasa vasorum and VEGF expression. One-way ANOVA test showed darapladib effect have significant differences (p<0.05) to total cholesterol lipid profile (p=0.049), HDL (p=0.000), LDL (p=0.000), amount of vasa vasorum (p=0.000), but it was not significant in VEGF expression (p=0.375). Darapladib administration had ability to decrease lipid profile and number of vasa vasorum. However, there was no significant effect in VEGF expression. This finding was probably due to another pathway besides VEGF mediated pathway. Further studies are required to understand compensatory pathways and their effects of atherosclerotic treatment.

1. Introduction
Cardiovascular disease or CVD accounts for 31% of worldwide deaths. More than 75% of CVD related death occurs in low income to moderate income countries, and 80% of all CVD related death are caused by cardiac arrest and stroke [1]. CVD has a close relation with atherosclerosis, Vessel wall (endothelium) dysfunction. Besides that, smoking, hypertension, obesity, stress, lack of physical activity, high cholesterol level, dyslipidemia and diabetes mellitus are factors which induce atherosclerotic pathogenesis [2].
The primary theory for atherosclerotic plaques development is oxidative stress. Decreased amounts of antioxidant concentration cause reduced ability to eradicate Reactive Oxygen Species (ROS) in the body. Furthermore, ROS induces angiogenesis. The increase levels of $H_2O_2$, HIF-1α, NF-κB, and iNOS in hypercholesterol presented by Angogenesis [3]. Early development of atherosclerotic plaque, endothelial dysfunction with increase in adhesion molecule exposure and adhesions of molecule on vessel wall has an effect on decreased production of nitric oxide, $O_2$, and other substances. Vasa vasorum of large arteries located on the tunica adventitia and tunica media supply oxygen and nutrition for arteries. Thickening of arterial wall in atherosclerosis causes hypoxia. During hypoxic state, Vascular Endothelial Growth Factor (VEGF) mediates the angiogenesis process induced by Hypoxia Inducible Factor-1 alpha (HIF-1α). Neo-vascularization of vasa vasorum in atherosclerotic animal model as a result of HIF-1α role where their level is regulated through oxygen dependent proteolysis HIF-1α mechanism [4].

In type 2 diabetes mellitus, significant levels of dense atherogenic Low Density Lipoprotein (dLDL) and Ox-LDL is present. Lp-PLA$_2$ hydrolyzes phospholipid oxidized from OxLDL. OxLDL produce oxidized non-esterified fatty acid (oxNEFA) and Lysophosphatidylcholine (LysoPC). LysoPC and oxNEFA have pro-inflammatory and atherogenic characters and are important contributors in diabetes mellitus atherosclerosis process [5].

Darapladib as a Lp-PLA2 inhibitor is a novel, selective, oral activated Lp-PLA2 inhibitor, and has clinical potential as a potent anti-atherosclerotic agent when administered in standardized combinations to treat patients with acute coronary heart disease [6,7]. Some research suggests that darapladib shows significant result in inhibiting the development of atherosclerosis (8) both in vitro (9) and in vivo test [10]. Several studies show darapladib is safe and may decrease cardiovascular disease incidence. Based on this background, this study to know darapladib effect on vasa vasorum formation and VEGF expression.

2. Materials and Methods

2.1 Darapladib
Darapladib was obtained from Glaxo Smith Kline USA in powder form. Darapladib was administered orally in 8 weeks and 16 weeks in High Fat Diet (HFD) groups with dosage 20 mg/body weight in kilograms.

2.2 Study Group
The total amount of sample used in this experiment is 30 male Sprague Dawley species rats weight 100-200 grams, age 6-8 weeks, healthy and no anatomic abnormalities. Each divided into three groups, normal with standard feeding diet standard feeding diet contained total energy calories 3.43 kcal/g (67% carbohydrate, 21% protein, an 12% fat), HFD group (total calorie energy of 5.29 consist of 58% fat, 17 carbohydrate and 25% protein) with type 2 diabetes mellitus, darapladib administration with HFD and type 2 diabetes mellitus. Each group then divided into two time series which were 8$^{th}$ week and 16 week.

2.3 Blood Glucose level, Plasma Insulin Level, and Insulin Resistance Measurement
Type 2 diabetes rat model was formed with HFD feeding and low dose STZ injection, 30 mg/bodyweight (in kilograms) intraperitoneal. Blood glucose level was measured using AccuCheck™ device. The blood was obtained from the rat-tail tip and then dripped onto the glucostick. Type 2 diabetes mellitus was diagnosed when blood glucose level of mouse model was above $>200$ mg/dL. Plasma level insulin measurement was done using ELISA sandwich method on Rat Insulin (Cat. No. E-EL R2466). Level of insulin obtained were ng/mL units. Plasma insulin conversion from ng/mL to IU/L was done using WHO formula by dividing it with 0.0347 because 1 IU is equivalent to 0.0347 mg/L. (11). Resistance of insulin measured by Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) formula. Rat models’ condition in blood glucose fasting and level of plasma insulin (12) was performed.
2.4 Lipid Profile Measurement
Lipid profile measurement was done by obtaining rat blood serum and using Kit EnzyChrom from BioAssay System USA.

2.5 Vasa Vasorum Measurement
Sample of the aortic tissue was surgically removed from mice as a measurement for vasa vasorum formation. Tissues were fixated using 10% formalin buffer overnight and then stained using Hematoxylin Eosin to observe vasa vasorum. Microscope with 400x magnification and version 2.4 dot Slide Olyvia software with the total of viewing field in each aortic specimen used to observed vasa vasorum. Vasa vasorum were characterized with luminal depiction in aortic layer which contain erythrocytes with pink colored center.

2.6 VEGF expression measurement
VEGF expression measurement was done after surgical removal of the aortic tissue of mice as a sample. Aortic tissue was fixated using PHEMO buffer for 10 minutes at room temperature. Blocking were done with 10% goat serum/PBS for 10 minutes and processed using double labelling immunofluorescene with HIF-1α anti rat antibody using FITC secondary antibody. Reading of VEGF expression was done using CLSM (Confocal Laser Scanning Microscope) and than measured quantitatively used Olympus Fluview software ver 1.7A.

2.7 Ethics
Ethically cleared with No.400/EC/KEPK/10/2016 has been granted by research ethics committee, Faculty of Medicine of Brawijaya University.

2.8 Statistical Analysis
The effect of darapladib administration towards lipid profile level, amount of vasa vasorum, and VEGF expression in rats with type 2 diabetes mellitus was analyzed using One way ANOVA with significant level of 0.05 (p=0.05) and 95% confidence interval (α=0.05). Furthermore, analysis was performed used Tukey HSD. Stastical test was conducted used SPSS 16 (IBM Cooperation, New York).

3. Result and Discussion
3.1 Blood Glucose Level, Plasma Insulin Level and Insulin Resistance
Rat model in type 2 diabetes mellitus defined used serial glucose of blood measurement, level of plasma insulin and insulin resistance. The highest mean value of blood glucose level occurred in 16th week diabetes mellitus (DM) group by 148 mg/ml. The highest mean value occurred in 16th week DM mouse was 11.2 IU/L. From HOMA-IR formula, insulin resistance 16th week DM mouse groups with the value of 2.789 (>1.716). In the 8th week DM mouse group, insulin resistance did not happen yet because the value has not exceeded the cut-off value. However, there was an increase in HOMA-IR value compared to other groups. This findings showed in 8th weeks DM rat group were approaching insulin resistance (Table 1).

3.2 Darapladib Treatment Effect on Lipid Profile
Significant effect of darapladib showed (p < 0.05) in low of lipid profile, cholesterol total (p=0.049), LDL (p=0.000) and HDL (p=0.000) as seen on Table 2. Darapladib have benefits both in lowering total cholesterol and LDL level. Darapladib has significant benefits in increasing HDL level.

3.3 Darapladib Treatment Effect to Vasa Vasorum Amount
With the administration of darapladib, there were lowering effects of the number of vasa vasorum was observed in 16th weeks DM groups with the mean value of 18 ± 5.891 cell/section (Table 2). Meanwhile, the lowest amount of vasa vasorum obtained from 8th week normal group has a mean value of 5 ± 1.581. One-way ANOVA test show Darapladib effect has a significant value (p<0.05) to the number of vasa vasorum (p=0.000). Darapladib presents decreased the amounts of vasa vasorum.

### Table 1. The value of blood sugar levels, levels of plasma insulin, and insulin resistance of each group

| Parameter       | Treatment Groups                                      |
|-----------------|-------------------------------------------------------|
|                 | Normal Diet 8 Weeks  | Diabetes Mellitus 8 Weeks | DM+Darapladib 8 Weeks | Normal Diet 16 Weeks | Diabetes Mellitus 16 Weeks | DM+Darapladib 16 Weeks |
| Fasting Blood Sugar Levels | (mg/ml) | 91.6±7.16 | 128±15.0 | 103.6±13.72 | 79.6±4.63 | 147.8±58.2 | 2 | 101.8±19.07 |
| (x±SD)          |            | 39 | 5.08±0.62 | 6.21±1.1 | 5.52±0.89 | 4.42±0.81 | 5.03±0.33 | 5.65±1.05 |
| Plasma Insulin Levels | (ng/mL) | 4.664±0.245 | 12.071±1.78 | 5.704±0.573 | 5.124±0.297 | 40.220±4.16 | 61 | 8.327±0.859 |
| (x±SD)          |            | 39 | 3.463±0.05 | 513 | 1.644±0.165 | 1.477±0.086 | 11.186±0.96 | 90 | 2.4±0.248 |
| Insulin Resistance | HOMA-IR VALUE (x±SD) | 0.486±0.067 | 1.551±0.496 | 0.647±0.141 | 0.462±0.079 | 3.789±0.41 | 7 | 0.954±0.142 |
| Interpretation | Normal | Normal | Normal | Normal | Normal | Insulin Resistance | Normal |

#### 3.4 Darapladib Treatment Effect to VEGF Expression

Darapladib administration showed no significant decrease in the VEGF expression in rat aortic tissue (Table 2). Highest VEGF expression was observed in 8th week DM group with the mean value of 1123.90 ± 184.39 AU. While, the lowest VEGF expression was obtained in 8th week normal group with the mean value of 809.395 ± 210.36 AU. Darapladib did not affect the decrease in VEGF expression significantly (p=0.375). Darapladib may decrease the VEGF expression but only in low small numbers.

Atherosclerosis is one of many cardiovascular diseases which increases in number at an alarming rate yearly. Hypercholesterol diet has significant association with atherosclerosis. Increasing cholesterol, TG and LDL levels play major role in atherogenesis [13]. High fat diet feeding in rats increases blood glucose level which induced type 2 diabetes mellitus [14]. Insulin receptor cells decrease due to down-regulation system event caused by high glucose in blood. If these conditions occur continuously and chronically, insulin receptor desensitization occurs simultaneously. It leads to a higher requirement of blood plasma insulin to incorporate glucose into cell tissue [15]. These findings constituents with Mawarti et al experiment in 2012. Their experiment proves that HFD feeding for 8 weeks to rat as a subject could induce insulin resistance and also induce a metabolic condition such as obesity seen in mouse [16]. If these conditions persist chronically, it could be insulin receptor desensitization. Hence, a higher plasma insulin level is needed in administering glucose in cell tissue. Mouse groups with insulin resistance 16th week DM groups with the value of 3.789 (> 1.716). In the 8th week DM groups, insulin resistance did not happen yet because the value did not exceed the cut-off value. However, there was an increase in HOMA-IR value compared to other groups. These findings showed that 8th weeks DM groups is approaching insulin resistance state.
Abnormalities in lipoprotein metabolism also relates with type 2 DM and triggers atherosclerotic pathogenesis [4].

Table 2. Parameters analysis by anova

| Parameter                        | Group of Treatment | P (ANOVA) |
|----------------------------------|--------------------|-----------|
|                                  | Normal Diet in 8 Weeks | Diabetes Mellitus in 8 Weeks | DM+Darapladib in 8 Weeks | Normal Diet in 16 Weeks | Diabetes Mellitus in 16 Weeks | DM+Darapladib in 16 Weeks | (ANOVA p<0.05) |
| Total Cholesterol of Lipid Profile (mg/dl) | 72.799±4.04 5 | 115.555±3.110 | 81.355±3.975 | 55.550±4.34 | 117.774±4.98 | 101.955±7.977 | 0.049 |
| HDL (mg/dl)                      | 34.739±8.31 2 | 8.354±2.0 57 | 20.017±0.355 | 35.757±1.575 | 18.145±0.889 | 21.400±5.057 | 0.000 |
| LDL/VLDL (mg/ml)                 | 48.831±5.05 5 | 88.195±3.075 | 50.337±2.543 | 19.241±1.545 | 102.142±15.545 | 55.509±11.330 | 0.000 |
| Number of Vasa Vasorum (cell/slide) | 5±1.581 | 15±3.391 | 11±4.930 | 7±2.345 | 18±5.891 | 8±2.967 | 0.000 |
| Expression of VEGF (au)          | 809.395 ± 210.36 | 1123.90 ± 184.39 | 1105.97 ± 184.09 | 1053.26 ± 61.45 | 1057.11 ± 345.94 | 1008.95 ± 211.88 | 0.375 |

Figure 1. Identification of Vasa Vasorum with Hematoxylin Eosin. (A) Normal Rats in 8 weeks. (B) Normal rats in 16 weeks. (C) Diabetes melitus rats in 8 weeks. (D) Diabetes melitus rats in 16 weeks. (E) Diabetes mellitus + darapladib rats in 8 weeks. (F) Diabetes mellitus + darapladib rats in 16 weeks. Magnification of 400x
High lipolysis processes cause a higher increase in free fatty acids level. In the liver, free fatty acid will be transformed to triglyceride Increased of VLDL is stimulated by high increase in triglyceride. A high plasma triglyceride and VLDL level induces a decreased of HDL and an increase of LDL. These conditions are known as dyslipidemia [17]. Based on the lipid profile measurement shown in the Table 2 mean total cholesterol level in type 2 DM groups with HFD feeding and type 2 DM groups with HFD feeding and darapladib administration are decreased after darapladib administration, both in 8th week or 16th week time series. The result also shows darapladib administration could increase HDL level, decrease total cholesterol level, and decrease LDL/VLDL levels.

![Image](image.png)

**Figure 2.** Immunofluorescence Result Of Vascular Endothelial Growth Factor (VEGF) Expression with FITC Using CLSM (Confocal Laser Scanning Microscope)

Hyper-cholesterolemia associated with endothelial dysfunction are involved in process of atherogenesis. Profile of lipid are worsen and dominated by stress oxidative. Thus, induces an imbalance in oxidant and anti-oxidant levels [18]. Stress oxidative in oxidized LDL to Ox-LDL caused by ROS. Oxidative stress causing oxidized LDL to easily bound to macrophage compared to non oxidized lipoprotein [19]. Tunica adventitia and tunica media of large arteries obtain their vascularization, oxygen and nutrition supply from vasa vasorum. Artery wall thickening in the atherosclerosis causes hypoxia. Oxidative stress in atherosclerosis produce H2O2 and inducing NF-kB activation through iNOS enhancement. Angiogenesis vasa vasorum mediated by VEGF and regulated by HIF-1α. During angiogenesis pathology, VEGF expressed by inflammatory cells and associated with atherosclerosis [3].

Recently, one of the lipid biomarkers associated with Lp-PLA2 have important role in atherosclerosis process [20]. Experimental data showed Lp-PLA2 have pro-inflammatory effect that influence the vessel wall [21]. Based on the data shown in Table 2, the amount of vasa vasorum from 8th week DMPD mouse group has the value of $11 \pm 4.930$ cell/section with the minimum value of 4 cell/section and the maximum value of 15 cell/section. 16th week DMPD mouse group has the mean value of $8 \pm 2.967$ cell/section with the minimum value of 5 cell/section and the maximum value of 13 cell/section. These findings showed that type 2 DM groups with darapladib adminstration has the lower amount of vasa vasorum compared to type 2 DM groups. During the 16th week DMPD group with their number of vasa vasorum approaching that in the 16th weeks normal group. This was caused by more effective darapladib administration during the 16th week compared to darapladib administration during the 8th week. However, darapladib administration for 8 weeks has the ability to decrease the amount of vasa vasorum if compared to 8 weeks administration in type 2 DM groups.
Besides the feeding category, age is also an important factor influencing the increase in vasa vasorum quantity. In the Table 2 showed that 16\textsuperscript{th} week DMPD group has a lower quantity compared to 8\textsuperscript{th} week DMPD group. Prolonged darapladib administration causes decreased of vasa vasorum over time. Based on one way ANOVA test, significant (p<0.005) decrease amount of vasa vasorum in 16\textsuperscript{th} week DMPD group shows there is an influence in the decrease amount of vasa vasorum in type 2 DM groups with 16 weeks darapladib administration. While, in 8\textsuperscript{th} week DMPD group significant difference is obtained from calculations, but no significant result was concluded from statistical analysis. A decrease caused by darapladib administration has the ability to inhibit Lp-PLA\textsubscript{2} formation, and also inhibit furthermore vasa vasorum angiogenesis.

However, VEGF administration based on the data shown in Table 2, darapladib administration effect on VEGF expression did not correlate with a decrease in their expression. In 8\textsuperscript{th} week DMPD group shows the mean value of 1105.97 ± 184.09 AU and in 16\textsuperscript{th} week DMPD group shows the mean value of 1008.95 ± 211.88 AU. Compared to the type 2 DM group, there is decrease of the expression between groups with darapladib administration (DMPD) both 8\textsuperscript{th} week and 16\textsuperscript{th} week time series. These results show type 2 DM group with darapladib administration correlates with a decrease in VEGF expression compared to type 2 DM mouse groups, but not influential. Based on one-way ANOVA, a significant decrease in VEGF expression was not found (p=0.375). VEGF expression in darapladib administration does not conclude a significant decrease, this probably caused by other unknown compensatory angiogenesis pathway than the primary VEGF pathway or there is a lack in experiment time frame to obtain a corresponding result.

Darapladib administration experiment with the dose 20 mg/body weight (in kilograms) in two time series, 8\textsuperscript{th} and 16\textsuperscript{th} week in type 2 Diabetes Mellitus rat group shows that darapladib could lower the number of vasa vasorum in the atherosclerosis process histologically. Darapladib has an important role as a Lp-PLA\textsubscript{2} inhibitor in the atherosclerosis process as measured from 2 vasa vasorum change parameters. However, VEGF expression does not show significant effects of darapladib administration. These results are estimated by other unknown angiogenesis pathway which did no mediated by VEGF. Further experiments are needed to search other pathway than the known VEGF mediated pathway, in their effect on atherosclerosis treatment.

4. Conclusion
Darapladib administration was able to decrease lipid profile and amount of vasa vasorum. However, there is no significant effect in VEGF expression. This findings were probably due to another pathway besides VEGF mediated pathway. Further studies are required to understand compensatory pathways and their effects on atherosclerotic treatment. This findings also showed in 16\textsuperscript{th} weeks Diabetes mellitus rat group without darapladib administration were approaching insulin resistance.

Acknowledgement
Faculty of Medicine Brawijaya University, Syiah Kuala University and Ministry of Research, Technology, and Higher Education.

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