Lp-PLA2 Selective Inhibitors (Darapladib) Effect in Lowering Serum and Aortic Lysophosphatidylcholine (LysoPC), NF-κB and Lp-PLA2 Levels on Dyslipidemic Rats Model

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Abstract

Background : Atherosclerosisis a chronicinflammatorydisease, and isassociatedwithupregulation of Lipoprotein-associated phospholipase A2 (Lp-PLA2), the enzyme thathydrolyzesphosphatidylcholine, producinglysophosphatidylcholine (LysoPC) and free fattyacids. LysoPCis a lipidmediatorwithknown pro-inflammatoryand pro-atherogenicproperties, and isbelieved to be a critical factor in cardiovasculardiseases.

Methods: Thirty male Rattus novergicus divided into three groups, those are Normal group: fed with rats food contained 3.44 kcal/g total calorie energy, Dyslipidemia group: fed with high-fat diet (HFD) contained 5.30 kcal/g total calorie energy and Dyslipidemia + Darapladib Group: fed with HFD and darapladib orally 20 mg/kg BW once a day. Each group consists of two serials treatment time: 8 weeks and 16 weeks. Expression of NF-κB, Lp-PLA2, and level of LysoPC aorta and serumwasthevariablethatmeasured.

Results: Darapladib has decreased the expression of Nuclear Factor Kappa B (NF-κB) ($p = 0.003$) and Lp-PLA2 ($p = 0.022$). Darapladib also decreased the level of LysoPC Serum on 8 weeks of serial treatment time ($p = 0.001$) but increased the level of LysoPC Serum on 16 weeks of serial treatment time ($p=0.040$). Pearson correlation test showed that serum and aortic tissue LysoPC level correlation was strong ($r = -0.584$) for 8 weeks of serial time and less strong ($r = -0.284$) for 16 weeks serial time.

Conclusion: Darapladib decreased expression of NF-κB, Lp-PLA2, and level of LysoPC serum on 8 weeks of serial treatment time but increased on 16 weeks of serial treatment time. Pearson correlation test showed that serum and aortic tissue LysoPC level correlation was an inverse correlation.

Keywords: *NF-κB, Dyslipidemia, LysoPC, Lp-PLA2*

Introduction

Enzyme Lipoprotein-associatedphospholipase A2 (Lp-PLA2) is a marker ofarterialplaqu edestabilizatio nandcancausep laquerupture, my ocardialischemia, and the ninfarction[1-3]. Enzyme Lp-PLA2 was also a markerofendo the lialdys function, which is the initial phase of atherosclerosis [4,5]. Low-density lipoprotein (LDL) will oxidizing and becomeox LDL[6,7]. Receptor Lp-PLA2 wasfound in oxLDL.Lp-PLA2 will bind to its receptor and hydrolyze group of acyl short on position phospholipids sn-2 of oxLDL formed two mediators lipid bioactive, namelyfatty acid oxidation (oxFA) lysophosphatidylcholine (LysoPC). LysoPCis a lipid mediator with known asproinflammatoryand pro-atherogenic agent. It is believed to be plays an important role in atherosclerosis underlying cardiovascular diseases and several pathological conditions are associated with elevated LysoPC levels in the circulation[8].

The inhibitor enzyme Lp-PLA2 agentisconsideredcapable of preventing ather oscleroticplaque formation[9]. There is more presence of active inflammatory cells and increased concentrations of Lp-PLA2 enzyme in unstable plaque, and that could be an important risk factor in the formation of atherosclerotic plaques and also in the process of the rupture[10].

NF-κB has an important role in the early stages of atherogenesis. NF-κB modified the initial LDL lipids and the formation of inflammatory mediators, including secretory phospholipase A2, 5-lipoxygenase, 12-lipoxygenase, and COX-2. NF-κB also influences inflammation that occurs in atherosclerosis through the cytokines they produce, such as TNF, IL-1β, IL-6, IL-10, IL-12,and interferon–γ[11].

Several studies prove that Lp-PLA2 is antiatherogenic and proatherogenic. In addition, it has developed preventive treatment of atherosclerosis through the Lp-PLA2 inhibitor. Based on the description above, it is very important to research to prove the provision of Darapladib as Lp-PLA2 inhibitor agents through the expression of Lp-PLA2, levels LysoPC tissue and serum, and the levels of the transcription factor NF-κB on dyslipidemia animal model divided in two serial times, that was 8 weeks and 16 weeks and also to describe the correlation of serum and aortic tissue LysoPC level. NF-κB has an important role in the early stages of atherogenesis. NF-κB modified the initial LDL lipids and the formation of inflammatory mediators, including secretory phospholipase A2, 5-lipoxygenase, 12-lipoxygenase, and COX-2. NF-κB also influences inflammation that occurs in atherosclerosis through the cytokines they produce, such as TNF, IL - 1β, IL-6, IL-10, IL-12, and interferon-γ.This study aims to prove the provision of darapladib as Lp-PLA2 inhibitor agents through the levels of LysoPC tissue and serum, the expression of NF-κB and Lp-PLA2, also to describe the correlation of serum and aortic tissue LysoPC level on early phase atherosclerosis.

Material and methods

Study Group

Thirty male Sprague-Dawley strain of *Rattus novergicus,* two months of age, and weight around 150-200 g were obtained from Bogor Agricultural University, Bogor, Indonesia, and bred at Bioscience Central Laboratory of Brawijaya University, Malang, Indonesia. These rats were divided into three main groups. Those are Normal group: fed with rats food contained 3.44 kcal/g total calorie energy (67% carbohydrate, 21% protein, and 12% fat), Dyslipidemia group: fed with high-fat diet contained 5.30 kcal/g total calorie energy (58% fat, 17% carbohydrate, and 25% protein) and Dyslipidemia + Darapladib Group: fed with high-fat diet and given darapladib (purchased from GlaxoSmithKline) orally 20 mg/kg BW once a day. Each group consists of 2 serials treatment time: 8 weeks and 16 weeks. The sample was sliced and stained in the Pathological Anatomy Laboratory, Faculty of Medicine, University of Brawijaya, Malang, Indonesia**.** Biochemical Parameters were measured at the Central Laboratory of Biological Sciences, Brawijaya University, Malang, Indonesia.

Animal ethical approval

Ethical approval was obtained for the experimental animal processes in this study from the Animal Veterinary and had Committee Brawijaya University License Number 400/EC/KEPK/10/2016.

Lipid Profile Measurement

Measurement of total cholesterol concentration, HDL, and LDL / VLDL was done by the calorimetric method by using EnzyChrom AF HDL and LDL/VLDL Assay Kit (cat number: E2HL-100,Gentaur, Belgium) [12,13].

Biochemical Test

The samples used were serum and aorta homogenates. Lp-PLA2 and NF-κB level were measured by immunofluorescence of aortic tissues that were previously fixated with PHEMO buffer and

were processed by imumunofluoresence labeling with antirat antibody Lp-PLA2 using rhodamin secondary antibody and anti-rat antibody NF-κB using fluorescein isothiocyanate (FITC) secondary antibody (BIOS Inc., Boston, MA, USA). These parameters were observedwith confocal laser scanning microscopy (Olympus Corporation, Tokyo,Japan) and were quantitatively analyzed using Olympus Fluo View software (version 1.7A; Olympus Corporation).LysoPC in serum level and aortic homogenate was measured with 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (16:0) (Avanti Polar Lipids, Inc., 700 Industrial Park Drive, Alabaster, Alabama, USA).

Statistical Analysis

Statistical methods used in this study were Kolmogorov-Smirnov for normality test and Levene for homogeneity test. Whereas, for the significance test, we used Oneway ANOVA to determine the effects of Lp-PLA2, LysoPC, and NF-κB expression in Sprague Dawley rats with dyslipidemia condition and treated by Darapladib. This analysis was continued by the *post* hoc test using the LSD method to detect the differences of parameters in each group. This study also used a correlation test to define the correlation of LysoPC aorta and Serum. Statistical analysis was performed with SPSS software version 20 (IBM Corporation, Armonk, NY, USA).

Results

Table 1. Measurement and analysis of Cholesterol Total, HDL, LDL/VLDL, LysoPC Aorta, LysoPC Serum, NF-κB Aorta and Lp-PLA2 in 8 weeks serial time

Group	Mean ± Standard Deviation						
	Cholesterol Total (mg/dl)	HDL (mg/dl)	LDL / VLDL (mg/dl)	LysoPC Aorta (ng/g)	LysoPC Serum (ng/mL)	NF-κB Aorta (aU)	Lp-PLA2 aorta (aU)
Normal Group	72.800 ±4.050	34.740±8.310	49.830 ±5.070	488159.4± 78.022	64445.2± 4767	1140.12±39,56 8	761.551 ± 19.087
Dyslipidemia Group	115.570 ± 3.100	8.360 ±2.070	88.200 ±3.080	324223± 87098	102750.6± 4662	1409.678±151, 311	1139.950 ± 55.565
Dyslipidemia and Darapladib Group	81.370 ±3.980	20.020 ±0.360	60.340 ±2.640	551355.6 ±99824	69 121.2 ± 1783	1104.656±54,2 67	1050.740 ± 27.857
P value	*0.048*	*0.000*	*0.001*	*0.010*	*0.001*	*0.003*	*0.022*

Data were presented as mean ± standard deviation (range) values. All the values of the parameters have been corrected into the International Standard of Mathematics (decimals).

*p <0.05 indicates a statistically significant difference.

ANOVA = Analysis of Variance; HDL = High Density of Lipoprotein; LDL = Low Density of Lipoprotein; VLDL = Very Low Density of Lipoprotein; LysoPC = lysophosphatidylcholine; NF-κB = Nuclear Factor Kappa Beta; Lp-PLA2 = Lipoprotein-associated phospholipase A2; PAI-1 = Plasminogen Activating Inhibitor–1; aU = arbitrary Unit

Table 2. Measurement and analysis of Cholesterol Total, HDL, LDL/VLDL, LysoPC Aorta, LysoPC Serum, NF-κB Aorta and Lp-PLA2 in 16 weeks serial time

Group	Mean ± StandardDeviation						
	Cholesterol Total (mg/dl)	HDL (mg/dl)	LDL / VLDL (mg/dl)	LysoPC Aorta (ng/g)	LysoPC Serum (ng/mL)	NF-κB Aorta (aU)	Lp-PLA Aorta (aU)
Normal Group	56.560 ±5.430	35.770 ±1.680	19.240 ±3.670	59838.6 ± 3038	107737 ± 15432	850.4 ± 56	823.3294 ± 56.323
Dyslipidemia Group	117.770 ±4.500	18.150 ±0.890	102.140 ±15.650	351501± 41338	110104.2± 4867	1322.8 ± 68	879.363 ± 65.220
Dyslipidemia and Darapladib Group	101.960 ±7.980	21.400 ±5.060	56.510 ±11.330	82042.4 6746	168838.2+10930	949.4 + 52	1129.570 ± 82.285
P value	*0.043*	*0.000*	*0.001*	*0.010*	*0.040*	*0,000*	*not significant*

Data were presented as mean ± standard deviation (range) values. All the values of the parameters have been corrected into the International Standard of Mathematics (decimals).

*p <0.05 indicates a statistically significant difference.

ANOVA = Analysis of Variance; HDL = High Density of Lipoprotein; LDL = Low Density of Lipoprotein; VLDL = Very Low Density of Lipoprotein; LysoPC = lysophosphatidylcholine; NF-κB = Nuclear Factor Kappa Beta; Lp-PLA2 = Lipoprotein-associated phospholipase A2; PAI-1 = Plasminogen Activating Inhibitor–1; aU = arbitrary Unit

Table 1 and Table 2 showed that it is a significant difference in lipid profile (cholesterol total, HDL, and LDL) between normal, Dyslipidemia, and Dyslipidemia + Darapladib Group on both serial treatment time ($p = 0.048$). This table was also showed a significant increase in NF-κB and Lp-PLA2 expression in dyslipidemia group and a significant decrease in NF-κB and Lp-PLA2 expression in dyslipidemia + Darapladib group on both serial treatment time ($p < 0.05$).

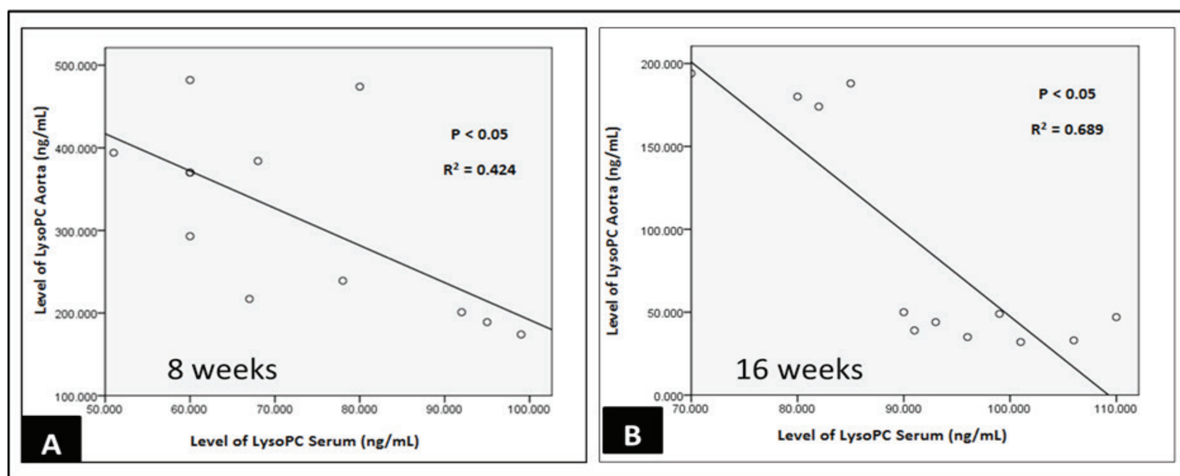

Figure 1. Linear regression of LysoPC serum and LysoPC Aorta Level on both 8 weeks (A) and 16 weeks (B) serial treatment time

Serum and aortic tissue LysoPC level correlation was an inverse correlation (Figure 1). The strength of serum and aortic tissue LysoPC level correlation was strong ($r = -0.651$, $r2 = 0.424$) for 8 weeks serial time and ($r = -0.83$, $r2 = -0.689$) for 16 weeks serial time.

There was a significant decreasing level of LysoPC Aorta ($p > 0,05$) but an increasing level of LysoPC Serum ($p = 0,01$) for 8 weeks of serial treatment time in Dyslipidemia Group. LysoPC Aorta and Serum levels were changed inversely at Dyslipidemia + Darapladib Group ($p < 0,05$). However, for 16 weeks of serial treatment time, this study showed a different result than 8 weeks of serial treatment time. In the dyslipidemia group, there was a significantly increased level of both LysoPC Aorta and LysoPC Serum ($p < 0,05$). On Dyslipidemia + Darapladib Group, LysoPC Aorta and Serum level were also changed inversely.Expression of NF-κB and Lp-PLA2, qualitatively observed using a confocal laser scanning microscope, can be seen in Figure 2.

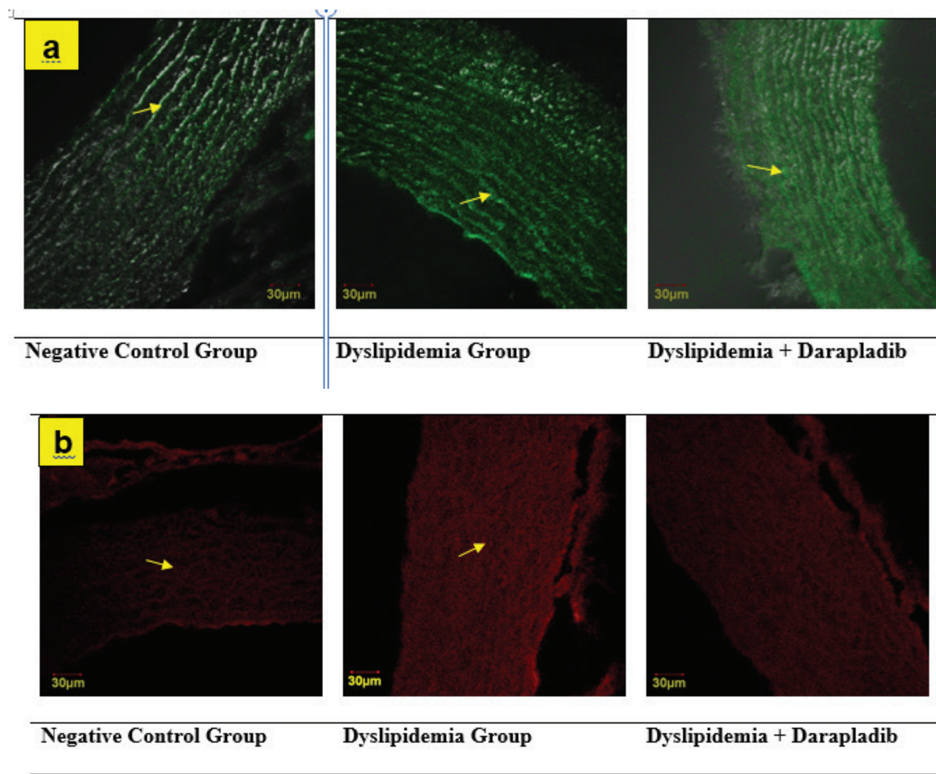

Figure 2. Qualitativeobservation of NF-κBandLp-PLA2 expression on aortic issue with immuno fluorescencestaining (yellow arrows): (a) NF-κBexpression; (b): Lp-PLA2 expression

Discussion

Lipid Fraction on High-Fat Diet Group

Lipid abnormalities fraction can be termed as dyslipidemia conditions. In the intestinal mucosal cells, free cholesterol is converted to cholesterol ester. Cholesterol ester joined to form VLDL triglycerides. VLDL then leave the heart and into the circulatory system. In the circulatory system, VLDL is converted to IDL by LPL and then into LDL. IDL and LDL are formed in the blood vessels can be entered into the endothelium of blood vessels or directly back to the liver. Ox-LDL phagocyted by macrophage cells(14).In macrophages, cholesterol esters hydrolyzed in to free cholesterol. Free cholesterol is brought to the surface of the cell membranes of macrophages and subsequently captured by HDL. Free cholesterol is converted into cholesterol esters and carried to the liver by HDL(15). This process will lead to the condition of Dyslipidemia

in conditions of a high-fat diet.

There is a close relationship between the Lp-PLA2 enzyme and LDL, as well as a comparable and inverse relationship with HDL[10]. However, this study shows that the combination of Lp-PLA2 inhibitors and lowering hypercholesterolemia drugs gives better results to reduce dyslipidemia. This is because Lp-PLA2 inhibitors provide additional substances that can reduce LDL[10].

Lp-PLA2 has a role in the pathogenesis of atherosclerosis and increases the risk of cardiovascular disease events[16]. Lp-PLA2 has been used as one of the early markers of atherosclerosis and as a therapeutic target [17,18]. Dyslipidemia conditions increase concentrations of LDL and VLDL in the plasma, therebyincreasingthelevels of Ox-LDL; fatty acids become oxidized fatty acids and will triggeran increase in freeradicals. Increased Ox-LDL and oxidized fatty acids will stimulate an increase in Lp-PLA2 activity to convert it into LysoPC [19,20]. Receptor Lp-PLA2 was found in oxLDL, and Lp-PLA2 will bind to its receptor and hydrolyze the group of acyl short on position phospholipidssn-2 of oxLDL, formed two mediators lipid bioactive namely lysophosphatidylcholine (LysoPC) and fatty acid oxidation (Oxidized Fatty Acid/oxFA) which had an important role in the process of atherosclerosis[21]. Those products have proinflammatory effects that initiate and increase the progression of atheroma.

Correlation Lysophosphatidylcholine (LysoPC) Level on aorta tissue and serum

Lysophosphatidylcholine (LysoPC) is one of the Lp-PLA2 enzyme product which has the chemical formula *[(2R) -2-hydroxy-3 - [(9z, 12z) -octadeca-9,12-dienoyl] oxypropyl] 2- (trimethylazaniumyl) ethyl phosphate* (National Center for Biotechnology Information. 2015). This study showedthatin Dyslipidemia Group, There was a significant decreasing level of LysoPC Aorta ($p > 0,05$) but an increasing level of LysoPC Serum ($p = 0,01$) for 8 weeks of serial treatment time. And LysoPC Aorta and Serum level were changed inversely at Dyslipidemia + Darapladib Group ($p < 0,05$). However, for 16 weeks of serial treatment time, this study showed different results than 8 weeks of serial treatment time. In dyslipidemia group, there was a significant increasing level both of LysoPC Aorta and LysoPC Serum ($p < 0,05$). On Dyslipidemia + Darapladib Group, LysoPC Aorta and Serum level were also changed inversely.

Serum and aortic tissue LysoPC level correlation was an inverse correlation. The strength of Serum and aortic tissue LysoPC level correlation was strong ($r = -0.651$, $r^2 = 0.424$) for 8 weeks serial time and ($r = -0.83$, $r^2 = -0.689$) for 16 weeks serial time. That means the increase of the LysoPC serum level will decreasing the levels of LysoPCAorta, and decreasing the LysoPC serum level will increase the levels of LysoPCaorta. This correlation and linear regression pattern were shown in Figure 1A (for 8 weeks of serial treatment time) and 1B (for 16 weeks of serial treatment time). Lysophosphatidylcholine (LysoPC) is the main product of Lp-PLA2 activity, which can induce the synthesis of proteoglycans and play an important role in the thickening of the intimal layer of the endothelium[22,23]. In several studies, this LysoPCcan stimulate proliferation apoptosis in endothelial and smooth muscle cells. LysoPC has also been instrumental in the recruitment ofmonocyte, leukocytes, and differentiation of T cells into the arterial wall[23,24].LysoPC is a chemotactic factor and led to the withdrawal of monocyte-macrophages to the endothelium and induce the expression of adhesion molecules in endothelial cells mononuclear resulted in the initiation of lesions increased[25].LysoPC performs its role in the pro-atherogenic processthrough this mechanism[26,27].

Nuclear Faktor Kappa Beta (NF-kB) expression and Lp-PLA2 on aorta tissue

Table 1 and Table 2 were showed a significant increase ($p < 0,05$) in NF-kB expression in Dyslipidemia Group and a significant decrease in NF-kB expression in Dyslipidemia + Darapladib group on both 8 weeks and 16 weeks of serial treatment time. Excessive expression of NF-kB aorta is on the tissue will be very influential in the pathogenesis of atherosclerosis[28]. In the early

stages of atherogenesis, NF-kB plays a role in the modification of the initial LDL lipids and the formation of inflammatory mediators, including secretory phospholipase A2, 5-lipoxygenase, 12-lipoxygenase, and COX-2. NF-kB also plays a role in the expression of monocyte chemoattractant protein-1 (MCP-1), which plays an important role in the migration of macrophages into the extracellular matrix of the tunica intima.

Active NF-kB is also able to regulate some adhesion molecules, including P-selectin, E-selection, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) [11,29]. After arriving in the extracellular matrix of the tunica intima, monocytes will soon turn into macrophages. This change involves a variety of macrophage colony-stimulating factor (CSF, M-CSF, or CSF-1). These factors are known to be regulated by NF-kB. This transcription factor also has a function in the regulation of matrix metalloproteinase-9 (MMP-9) were able to degrade the extracellular matrix that allows the migration of macrophages into the tissue. NF-kB also influences inflammation that occurs in atherosclerosis through the cytokines they produce, such as TNF, IL-1β, IL-6, IL-10, IL-12, and interferon-γ[28,30].NF-kB is a protein complex that works as a DNA transcription controller. The protein is involved in cellular responses to external stimuli such as free radicals, ultraviolet radiation, oxidized LDL, stress, cytokines, and bacterial or viral antigens[31]. Therapy with LpPLA2 inhibitor has succeeded in reducing the expression of NF-kB. NF-kB canonical pathway stimulated by reactive oxygen species (reactive oxygen species / ROS), lipopolysaccharide, and the signal from cytokine receptors such as TNF, IL-1, and Toll-like receptors. NF-kB pathway activated by specific proteins of the TNF family, such as lymphotoxin β, factor B cell activation, and CD40 ligand. Stimulation of these proteins causes the translocation of NF-kB complex / RelB: p52 into the nucleus[32].

Table 1 (for 8 weeks) and Table 2 (for 16 weeks) were showed a significant increase ($p < 0,05$) in Lp-PLA2 expression in Dyslipidemia Group and significantly decreasing Lp-PLA2 expression in Dyslipidemia + Darapladib group on both 8 weeksand 16 weeks of serial treatment time.

Lp-PLA2 is a single chain glycoprotein member of the serine protease inhibitors (or serpins) family. Endothelial cells, smooth muscle cells, fibroblasts, monocytes/macrophages, adipocytes, endometrium, peritoneum, liver cells, mesothelial cells and cardiac myocytes could produce Lp-PLA2. Lp-PLA2 is a major stress-induced gene. Once synthesized, Lp-PLA2 is mainly stored in platelets and secreted to blood flow or deposited on the subendothelial matrix. The increased expression of Lp-PLA2 *in vivo* suppresses fibrinolysis, consequently leading to the pathological fibrin deposition and then could cause tissue damage[33]. Inflammatory cytokines (IL-6, IL-1, TNF-α), growth factors, and hormones (insulin, glucocorticoids)also influence Lp-PLA2. Chronic inflammation, insulin resistance, and obesity influenced the expression of Lp-PLA2. Several studies had been demonstrated that fibrinolytic dysfunction (defined by Lp-PLA2 levels) mediates the increased risk of CVD in individuals with metabolic syndrome, and increased Lp-PLA2 concentrations have been found both in blood and in coronary plaques of metabolic syndrome patients[34].

Conclusion

Lp-PLA2 inhibitor (darapladib) has affected a fraction of lipids (LDL / VLDL and increase HDL) and decreased NF-kB and Lp-PLA2 expression on both 8 weeks and 16 weeks of serial treatment time. Lp-PLA2 inhibitor (darapladib) also decreased the level of LysoPC Serum on 8 weeks of serial treatment time but increased the level of LysoPC Serum on 16 weeks of serial treatment time. Serum and aortic tissue LysoPC level correlation was an inverse correlation. That means the increase of the LysoPC serum level will decreasing the levels of LysoPC Aorta, and decreasing the LysoPC Serum level will increase the levels of LysoPC Aorta. The strength of Serum and Aortic tissue LysoPC level correlation was strong for 8 weeks of serial treatment time and less strong for 16 weeks of serial treatment time.

Conflict of Interest: The authors declare no conflict interest

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