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Clinical Research

Three Dangerous Loops of Lipoprotein-Associated Phospholipase A2 Activity on Increasing LDL Atherogenecity

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Abstract

Background: Hypercholesterolemia is a major classic risk factor for cardiovascular disease; however, there are 35%-40% cases of cardiovascular where patients have normal cholesterol levels. Lp-PLA2 is an enzyme that is produced and secreted by macrophages as a response to the lipid peroxide formation, especially the platelet activating factor compound and phosphocholine peroxide. Lp-PLA2 has been correlated with classic risk factor of cardiovascular disease, although that correlation with number of foam cell at early stage of atherosclerosis is not clear yet. This study aims to determine the lipid profiles, oxidation stress markers and Lp-PLA2 levels at three different initial atherogenesis levels.

Methods: This study observed the change of Lp-PLA2, F2-Isp, MDA, TC, LDL, HDL levels in rat serum at three different levels of early atherogenesis; they were Ath-I, Ath-II and Ath-III made on the number of foam cells. The number of cells was observed in all aortic cross sectional surfaces, using the Oil-Red-O staining. The LDL-C content was measured using the Fiedwall formula, whereas the MDA content was measure by using TBA-test. The observation of F2-isoprostane and Lp-PLA2 were exemplified by the procedure of Elisa's.

Results: The one way ANOVA test results between the three initial levels of atherosclerosis showed no significant differences in all lipid profiles both in serum and stress oxidation markers. However, the LSD test results projected significant differences in LDL levels in Ath-I compared to others. There was a significant difference ($p<0.01$) in the serum of Lp-PLA2 content. The LSD test results displayed a significant increase in Lp-PLA2 enzyme levels since the Ath-II stage.

Conclussion: The elevated levels of Lp-PLA2 also increased the atherogenecity of LDL, due to the increased inflammation, stress oxidation and elevated levels of Lp-PLA2, which were interconnected with proatherogenic loops.

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Key words: Atherogenesis, Foam Cells, F2-Isp, LDL, Lp-PLA2, MDA

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Introduction

The process of atherogenesis begins with the formation of foam cells at the layer of sub-intima blood vessel walls. Atherosclerosis is the main cause of morbidity and mortality in the developing countries and becomes the primary etiology of cardio vascular diseases (CVD) such as coronary heart Disease (CHD), *coronary arterial disease* (CAD) dan cerebro vascular disease. Most CHDs are in the form of heart attack and most ischemia

cases occurring in the brain are secondary effects of atherosclerosis[1,2].

Atherosclerosis has many risk factors, among others: old age, smoking habit, obesity, hyper cholesterol, diabetes and high blood pressure. Hyper cholesterol is the main classical risk factor of atherosclerosis[4] in addition to the age factor[2], since 30-40% part of the atherosclerosis plaque is made from crystal cholesterol, ether cholesterol and lipid peroxide[3,5]. Oxidized Low Density Lipoprotein (oxLDL) instead of native Low Density Lipoprotein (nLDL) plays a major role in developing atherosclerosis from the early to the advanced stages, where an oxLDL plasma is expected to be able to be used as the marker in diagnosing atherosclerosis6. In contracts, a controversy has been identified. Hyper cholesterols, which was the main risk factor of atherosclerosis with the data in the field showing that 50% patients with atherosclerosis complication such as myocardial infarction did not have the classical risk factor[7,8] and in 35-40% of all CHD cases, the patients turned out showing that the total cholesterol content to be normal[4,9]. Therefore, a study on nonconventional biomarkers which mostly are found in serum but are closely related to the process of atherogenesis, especially at early stages that may improve proterogenic effects of LDL at the normal content during the early stage of atherosclerosis, is very important.

A lipoprotein-associated phospholipase A2 (Lp-PLA2) enzyme which is also called the platelet activating factor of asetilhydrolase (PAF-AH) is an enzyme produced and secreted by macrophages as a response to the lipid peroxide formation, especially the PAF compound and phosphocholine peroxide[10]. In the circulation, Lp-PLA2 is bound with apoB of the LDL[11].

Various classical risk factors of atherosclerosis are positively correlated with the content of Lp-PLA2 plasma, such as hypercholesterol[12], diabetes[13,14], hypertension[15], metabolic syndromme1[3,16], as well as symptomatic carotid atherosclerotic plaque17,18. The hypolipidemic drug decreases LP-PLA2 activity and concentration[19]. Epidemiologic data show that Lp-PLA2 influences the cardiovascular disease (CVD), either at individuals' hypercholesterol and normal cholesterol[20,21] levels, yet its role and correlation with various risks factors and critical value of atherogenic Lp-PLA2 at the preliminary level have not been further studied.

Atherosclerosis is a chronic inflammation with lipid deposits in inflammatory cells, involving oxidative stress and endothelial dysfunction. The oxidized LDL (oxLDL) is a substrate of the Lp-PLA2 enzyme. As the Lp-PLA2 substrate, oxLDL can stimulate the expression and secretion of Lp-PLA2[11,22] so that Lp-PLA2 is also classified as an oxidative stress marker23. Malonyl dialdehyde (MDA) and F2-Isoprostan (F2-Isp) are known as common markers of oxidative stress because they are not only generated from arachidonic acid metabolism through the cyclooxygenase pathway, but also act as a lipid peroxide of linoleic acid and docohexanoic acid[24]. MDA and F2-Isp can be found in tissues as well as in plasma. F2-isoprostan is the best in vivo oxidation marker to date, because it is noninvasive and has high specificity[25]. People with atherosclerosis risk factors have shown an increase in F2-Isp include hypercholesterolemia[26], diabetes[25] and hypertension[27]. Like Lp-PLA2, measurements of MDA and F2-Isp levels in plasma in advanced atherosclerosis also portrays a significant increase[26,28,29,30]. Observation in rats with combination of hyper cholesterol feeding treatment and duration of eating reported that Lp-PLA2 aorta was not significantly different. However, serum levels of Lp-PLA2 indicated significant differences. In addition, the rate of serum elevation has occurred earlier than the increase in the number of foam cells[31].

Based on previous research results, it is necessary to conduct further study on the harmful loops caused by Lp-PLA2 serum activity in the early stages of atherogenesis, depending on the different number of foam cells. This present research is intended to observe the changes the Lp-PLA2 enzyme contents, lipid profiles, and oxidation stress in the serum at the different stages of atherogenesis, as shown at the different number of foam cells resulted by high lipid diet in different times.

Methods

Animal Housing. The selected experimental animals in this research were male rats (*Rattus norvegicus*) with the age of 6-8 weeks, and the weight of 150-200 grams. The rats were obtained from CV. Gamma Scientific Biolab Malang. The materials for making hyperlipidemic foods were BR1(45%, P.T. Wonokoyo Corporation), cholesterol (2%, sigma Aldrich), folic acid (0,2%, sigma

Aldrich), lard (7,5%, extracted from fresh pork adipose tissue), butter (5%) and wheat flour (22,5%, CV. Bogasari). The animals were kept under 12-hour bright and 12-hour dark conditions, and foods and drinks were given in ad libitum. This research had obtained an ethical clearance from the Medical Faculty, Universitas Brawijaya, Malang, Indonesia, No. 0313/EC/KEPK-S3-JK/11/2010).

Experimental Design. In this research, a Post Test Control Group Design was employed. Thirty rats at the age of 6-8 weeks with each weight of 150-200 grams were divided into 2 groups, where the control group were given normal foods (BR1) and the experimental one with the hyperlipidemic foods for 2, 8 and 12 weeks periods (5 rats for each period), in order to obtain variations in the atherosclerosis development. At the end of the treatment, the serum of lipid profiles (covering the contents of the total cholesterol (TC), Triglycerides (TG), Low Density Lipoprotein (LDL), High Density Lipoprotein (HDL), Lipoprotein phospholipase A2 (LpPLA2), F2-Isoprostane (F2-Isp), Malonil Dealdehid (MDA) and the number of foam cells (FC) in aorta tissue were investigated. The data on the number of foam cells were made in three different levels of atherogenesis, namely atherogenesis level 1 (Ath-I), level 2 (Ath-II) and level 3 (Ath-III).

Sample Preparation and Assay Variable in Serum. At the end of the treatment, in order to extract the blood and blood vessels, the rats were anesthetized. The blood sample was taken from the heart, and then centrifuged in 3000 rpm for 10 minutes. The serums produced were soon kept under the temperature of -20ºC before being used. The LDL-C content was measured with the Fiedwall formula; the MDA with the TBA test method were observed using a spectrophotometer with the wave length of 530.6 nm. Further, the F2-Isp were examined using F2-isoprostant Immunoassay Kit (Direct 8-iso-Prostaglandin Fά Enzyme Immunoassay Kit, Assay Design with the catalog number of 900-901). The observation followed the procedures stated in the product and was made using a spectrophotometer with the wavelength of 405 nm. Lp-PLA2 was measured using the Sandwhich Ellisa method, examined with Elisa reader at the λ 450 nm by following the procedure stated in the product (LpPLA2 Sandwich Elisa Kit produced by Cusabio Biotech CO., Ltd., with catalog number of CSB-E08320r).

Preparing the Aorta Tissue and Foam Cells Examination. The aorta blood vessels near the heart were cut for 1.5 cm and were washed using PBS, then were kept in the freezer at the -20ºC to make preparation of the fresh frozen section. The frozen aorta were *cross-cut* with the thickness of 5 μm using cryo cut. The prepared materials were kept at -4ºC and then stained with RedO and HE Oil. Examination was made on one cycle of the *cross-cut* aorta using the light microscope with the magnification of 40x10.

Statistical Analysis. A one way ANOVA test was employed to test the effects of the atherogenesis levels on all variables in the serums (the contents of TC, TG, LDL, HDL, F2-Isp, MDA and Lp-PLA2). A Least Significant Difference (LSD) test was exercised to know the pair-wise comparison of the treatment's mean with or without significant difference. The ANOVA and LSD tests were done using SPSS 16.0 computation software.

Table 1. Lipid Profiles in the Rat's Blood Serums at Various Stages of Atherogenesis

Stages of atherogenesis, Cells/ field of view)	TC±STDEV (mg/dL)	TG ± STDEV (mg/dL)	LDL±STDEV (mg/dL	HDL±STDEV (mg/dL)
Ath-I	125.11±20.77	110.44±19.26	74.33±21.03	28.33±5.33
Ath-II	148.19±29.49	121.10±16.76	97.25±27.82	26.75±3.29
Ath-III	158.40±32.84	128.60±18.99	106.88±28.39	25.80±2.05
Anova test	p>0.05	p>0.05	p>0.05	p>0.05
		LSD test		
Ath-I vs Ath-II	p>0.05	p>0.05	p<0.05	p>0.05
Ath-I vs Ath-III	p<0.05	p>0.05	p<0.05	p>0.05
Ath-II vs Ath III	p>0.05	p>0.05	p>0.05	p>0.05

non-significant ($p>0.05$); significant ($p<0.05$); very-significant ($p<0.01$)

Table 2. F2-Isp, MDA and of Lp-PLA2 Profiles in the Rat's Blood Serum at Various Stages of Atherogenesis

Stages of atherogenesis, Cells/ field of view)	Mean Content of the F2-Isp Serum ± STDEV (ng/mL)	Mean Content of MDA Serum ± STDEV (ng/mL)	Mean Content of Lp-PLA2 Serum ± STDEV (ng/mL)
Ath-I	172.38 ±38.19	2.388 ± 0.512	158.81 ± 50.10
Ath-II	188.98 ± 45.02	2.756 ± 1.567	212.28 ± 44.43
Ath-III	218.15 ± 33.37	3.618 ± 2.052	255.14 ± 28.03
Anova Test	p>0.05	p>0.05	p<0.01
	LSD Test		
Ath-I vs Ath-II	p>0.05	p>0.05	p<0.01
Ath-I vs Ath-III	p>0.05	p>0.05	p<0.01
Ath-II vs Ath III	p>0.05	p>0.05	p>0.05

non-significant (p>0.05); significant (p<0.05); very-significant (p<0.01)

Results

The content of Lipid profile, oxidation level of LpPLA2 serum enzyme at different atherogenic levels

The number of foam cells observed in the frozen slices of blood vessels in a full circle has a range between 2-146 foam cells. From these data, three different levels of atherogenesis were identified, they were categorized into level 1 (Ath-I, less than 50 foam cells, 17.33±11.22 cells), level 2 (Ath-II, between 50 to less than 100 foam cells, 68.00±13.64 cells) and level 3 (Ath-III, more than 100 foam cells, 124.2±18.50 cells). The ANOVA test results showed that the number of foam cells at the three levels of atherosclerosis differed very significantly (p< 0.01), as well as the results of all mean comparisons at the three levels of atherosclerosis using the LSD test.

One way ANOVA and LSD test results showed that all TC, TG and HDL lipid profile contents did not show any significant difference (p<0.05) among the three levels of atherogenesis. While the results of statistical tests on LDL levels showed that, even though the ANOVA test results did not show a significant difference, however LSD test showed that Ath-I was significantly different (p<0.05) from the others (Table 1). One way ANOVA and LSD test also did not identify differences in oxidation stress levels on MDA or F2-Isp parameters (p>0.05) among the three levels of atherogenesis. ANOVA test of LpPLA2 level in serum projected a very significant difference (p<0.01). It was in accordance with the LSD test results that displayed a significant difference between the contents of Lp-PLA2 in the Ath-I, and those of Ath-II and Ath-III. However, no significant difference occurred between Ath-II and of Ath-III (Table 2).

Discussion

The three levels of atherogenesis in this study showed no significant difference in lipid profiles. This was due to the fact that these three stages are the early stages of atherogenesis. However, the three levels of atherogenesis show a tendency that there is an increase in lipid profile of TC, TG and LDL. The LSD test results projected that the LDL content in Ath-III was significantly higher than Ath-I and Ath-II. The HDL content inclined to be inversely proportional to the levels of atherogenesis. This is also the case of oxidative stress levels indicated by the tendency of elevated levels of F2-Isp and MDA along with the increased levels of atherogenesis in blood vessels.

It cannot be denied that the material deposited in blood vessel walls in foam cells and atheroslerotic plaque in individuals with normal cholesterol or hypercholesterolemia are cholesterol molecules. Therefore, it can be ascertained that this is another factor which increases the cholesterol reactivity in the blood circulation so as to trigger an atherogenic inflammatory reaction even if the contents are normal. Among the parameters, only Lp-PLA2 serum content that has shown significant difference since the initial stage and the content up to stage of Ath-II has been stabilized in this study. The content of Lp-PLA2 in the serum of 212.28 ± 44.43ng/ml (Ath-II) has been identified significantly different from that of the Ath-I (158.81 ±

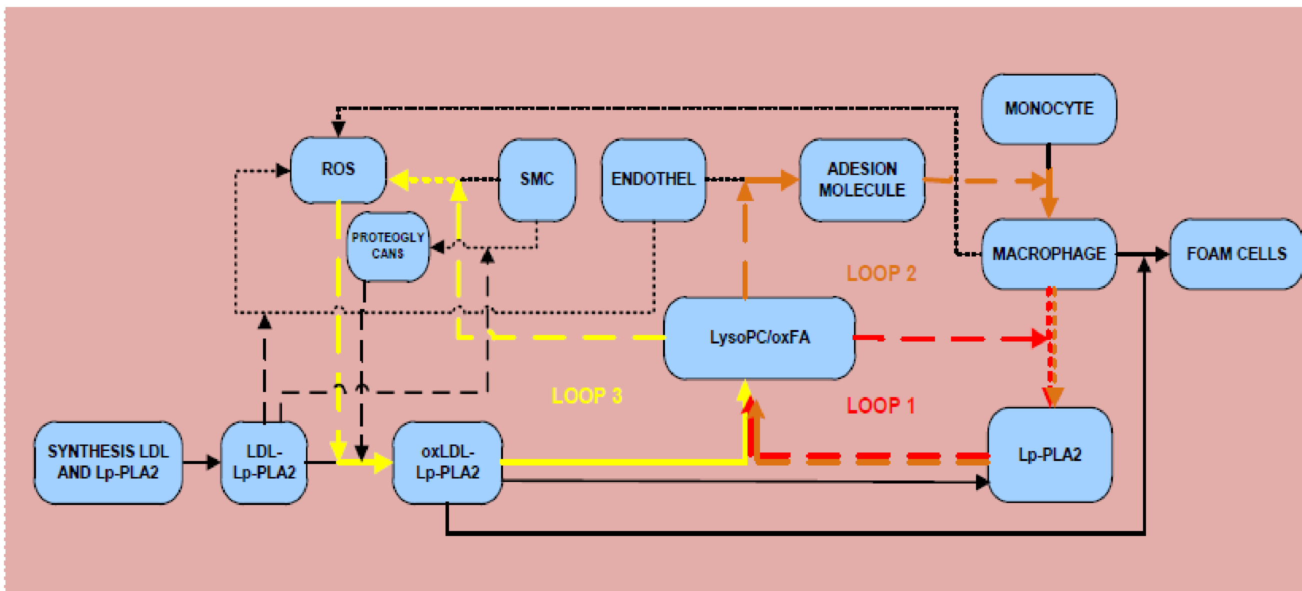

Figure 1. Three Dangerous Proatherogenic Loops caused by Lp-PLA2 Enzyme
Notes:
LDL: Low Density Lipoprotein, Lp-PLA2: Lipoprotein-Associated Phospholipase A2, LDL-Lp-PLA2: Complex LDL-Lp-PLA2. oxLDL-Lp- PLA2: Complex LDL-Lp-PLA2 Oxidized, LysoPC: Lyso Phosphatidylcholin, oxFA: Oxidized Fatty Acid, oxLDL: Oxidized Low Density Lipoprotein, oxPC: Oxidized-Phosphatidylcholine, ROS: Radical Oxygen Species, SMC: Smooth Muscle Cell

50.10 ng/ml) and it increased at the Ath-III (255.14 ± 28.03). This condition due to the possibility of the Lp-PLA2 enzyme activity which was able to improve the characteristic of the atherogenecity of the LDL cholesterol so that it is deposited at the blood vessel walls even though the cholesterol content in the serum was still at the normal category.

A high increase was found in the content of Lp-PLA2 serum above 200 ng/ml at Ath-II in the observation of atherogenesis and a relatively stable increase and then a slowly increase at the next period though the addition in the content was not significantly different. At the Ath-III period, it was projected that the content has been able to push ahead a significant level of atherogenesis. It might be that the content of Lp-PLA2 would rise to the next level of atherogenesis to reach its maximal content. A similar phenomenon also happened to the research by Shi et al. using experimental animal pigs with diabetes/ hypercholesterol11. The content of Lp-PLA2 serum in the research had increased sharply since the observation at the fourth week and was still high without any significant increase at the observations from the 12th to the 24th weeks.

In regards to the materials deposited either in the foam cells or atherosclerotic plaques that were LDL, the increase of the differences in the number of foam cells occurred more rapidly than the increase in the LDL. The Lp-PLA2 as a lipase enzyme plays a functionally important role in the process of atherogenesis as it is closely related to LDL.

Lp-PLA2 levels correlated with LDL levels and both correlated with the number of foam cells either directly or indirectly. Thus, it is predictable that the enzyme of Lp-PLA2 becomes the key molecule for the process of aterogenesis carrier of LDL accumulation in the foam cell. This condition also in line with Goncalves's et.al's finding, suggesting that the Lp-PLA2 level is significantly determinative in correlation with LysoPC levels as well as inflammatory cytokine32. The results of this study indicated no correlation between formation of foam cells with both oxidation stress markers; MDA and F2-Isp. The findings suggested that oxidative stress acted not directly on foam cells formation, but depended on LpPLA2 enzyme activity. This is consistent with the finding of Stafforini's et.al that showed the release of F2-Isp from esterified phospholipids that was also catalyzed by both intracellular and plasma Lp-PLA2[33].

The potential of Lp-PLA2 as the promoter of LDL atherogenesis was not only supported by this research results, but also by various other researches. If observed in detail, Lp-PLA2 showed its role in the whole process of atherogenesis that might cause the three dangerous pro-atherogenic loops (Figure 1).

Phosphatydilcholine (PC) is a main phospholipid, the composer of cell membrane and LDL[34]. Oxidation by free radicals to oxLDL results in oxPC that becomes the substrate of the Lp-PLA2 enzyme activities. Lp-PLA2 is bound to be apoB of LDL11 (Shi et al. 2007), hydrolyzing an oxPC to produce LisoPC and oxFA[21,35]. In an analogy of a production machine and the foam cells as the product of atherogenesis, Lyso-PC causes the formation of three dangerous proatherogenic loops that may be categorized into 3 loops:

Loop 1. The activator of the atherogenesis process (positive feedback of Lp-PLA2 production)

LysoPC induced the macrophages so that it produced inflamatory cytocin of TNF-α, IL-6 and IL-1β, playing the role as an autocrine as well as stimulating macrophages to produce Lp-PLA2 [11,32].

Loop 2. The operator of the atherogenesis process (Positive feedback of proatherogenic inflammatory reaction)

LysoPC stimulated the endothel to excrete its adhesive molecules[21,36,37] which then facilitated the migrate of monocytes from the circulation to sub-endothel, assisting the development of monicytes into macrophages and foam cells[11].

Loop 3. The generator of the atherogenesis process (positive feedback of ROS production).

In addition to its role as the source of Lp-PLA2, macrophages also acted as potential sources of ROS so that the formation reaction of oxLDL and oxPC as the substrate of Lp-PLA2 might produce lysoPC as the derivative actor for the Proatherogenic to continually occur. LysoPC also stimulated SMC of blood vessel walls to produce ROS37. The three loops above have facilitated the occurrence of LDL oxidation, inflammation reaction, ROS formation and provision of Lp-PLA2 enzyme which all are keys to form foam cells and to support atherogenesis to continue to happen although the LDL content was at the normal level.

Conclussion

Studies have shown that the presence of the Lp-PLA2 serum can be used as a basis to explain the presence of CVD patients with normal cholesterol but with increasing Lp-PLA2 enzyme. The presence of Lp-PLA2 significantly improves proatherogenic characteristic of LDL. The serum content of Lp-PLA2 for more than 200ng/ml is a critical content that can improve the atherogenic characteristic of LDL. The increased levels of Lp-PLA2 has caused an increased atherogenecity of LDL, due to the increased inflammation, stress oxidation and elevated levels of Lp-PLA2, which act as the proatherogenic factors.

Ethical Clearance

This study was approved by the institution's ethic committee.

Conflict Of Interest

None

Publication Agreement

The authors of this article give permission to Indonesian Journal of Cardiology to publish this article if this article is accepted

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List of Abbreviations

CAD: Coronary Artery Disease
CVD: Cardiovascular Diseases
F2-Isp: F2-Isoprostane
FC: Foam Cells
HDL: High Density Lipoprotein
PAF-AH: platelet activating factor of asetilhydrolase
LDL: Low Density Lipoprotein
Lp-PLA2: lipoprotein-associated phospholipase A2

MDA: Malonyl Dialdehyde
nLDL: native Low Density Lipoprotein
oxLDL: Oxidized Low Density Lipoprotein

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