

DECREASEMENT OF LYSOPHOSPHATIDYLCHOLINE LEVEL, NUCLEAR FACTOR KAPPA B EXPRESSION, INTIMA-MEDIA THICKNESS AND IMPROVEMENT OF INSULIN RESISTANCE BY DARAPLADIB TREATMENT: *IN VIVO* STUDIES OF TYPE 2 DIABETES MELLITUS SPRAGUE-DAWLEY RAT MODEL

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ABSTRACT

Objective: Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is an enzyme with several pro-inflammatory properties that involved in pathogenesis of atherosclerosis, but some investigation shows controversial views regarding its biological role. We examined the effect of selective inhibitor of Lp-PLA₂ (darapladib) to the inflammation marker, intima-media thickness (IMT), and insulin resistance (IR) of type 2 diabetes mellitus (T2DM) rat model. This study aimed to measure lysophosphatidylcholine (lyso-PC) in serum and aortic tissue, nuclear factor kappa B (NF-κB) expression, IMT, and IR with darapladib treatment in a T2DM rat model.

Methods: 30 Sprague-Dawley rats were randomly divided into normal group, T2DM group and T2DM with darapladib treatment. Induction of T2DM was done by giving high-fat diet and low dose injection of streptozotocin. Blood glucose level and insulin plasma concentration were measured to calculate IR. 8 weeks and 16 weeks after treatment, we compared lyso-PC level, NF-κB expression, and IMT.

Results: Darapladib significantly decreased lyso-PC level, NF-κB expression, and IMT at two serial treatments. Darapladib treatment group exhibited significant reduction of IR (0.64±0.11 vs. 2.07±0.16, at 8 weeks; and 0.93±0.08 vs. 6.48±0.55 at 16 weeks) compared with T2DM group.

Conclusions: These data suggested that Lp-PLA₂ played a role in inflammation process, atherosclerosis, and IR occurring in metabolic disorder.

Keywords: Type 2 diabetes mellitus, Inflammation, Insulin resistance, Atherosclerosis, Lipoprotein-associated phospholipase A₂.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is one of the most noncommunicable diseases involving multiple genes and environmental factors that characterized by impaired insulin secretion through a dysfunction of pancreatic β-cells and insulin resistance (IR) [1]. It is associated with an increased risk of atherosclerotic cardiovascular disease [2]. Prolonged exposure to hyperglycemia altered vascular homeostasis and associated with vascular inflammation that has been hypothesized underlying atherosclerosis process in T2DM condition [3]. Both free fatty acid and AGE directly activate nuclear factor kappa B (NF-κB) that required for the transcription of most pro-inflammatory molecules, adhesion molecules, cytokine in the pathology of atherosclerosis, and diabetic complication [4,5]. The American Heart Association recommends intima-media thickness (IMT) measurement to estimate atherosclerotic events in cardiovascular disease. Carotid artery represents the condition in coronary artery disease, so IMT was widely used as intervention in the atherosclerotic management.

The secreted plasma form of platelet-activating factor acetylhydrolase (PAF-AH) also known as lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is an enzyme circulates in blood and found in atherosclerotic lesion. This enzyme has a dualism since it has anti-inflammatory properties to hydrolyze pro-inflammatory glycerophospholipid PAF and has pro-inflammatory properties since its substrate generates lysophosphatidylcholine (Lyso-PC). Lp-PLA₂'s pathways that related to inflammation have been hypothesized to have a role in atherosclerosis process. Darapladib is a reversible Lp-PLA₂ inhibitor that has been extensively tested in vitro and in vivo [6]. However, studies show

controversial results about Lp-PLA₂'s role in cardiovascular disease [7]. Hence, this study aims to determine the role of Lp-PLA₂ selective inhibitor to the lyso-PC level, NF-κB expression, IMT, and IR in T2DM rat model.

METHODS

The study was carried out in the Central Laboratory of Biological Sciences, Brawijaya University Malang, Indonesia. The investigation was conducted according to the principle expressed in the "Guiding Principles in the Care and Use of Animals." The study received prior approval from the ethics committee of the Animal Care and Use Committee Brawijaya University Number 229-KEP-UB.

Animals

Thirty male, 6-week-old Sprague-Dawley rats (Bogor Agricultural University, Indonesia), 150-200 g weight each were divided into negative control (normal) group, positive control group (T2DM), and study group (T2DM with darapladib treatment). Each group was divided into two serial treatments: 8 weeks and 16 weeks. The negative control group was given normal rats food contained total energy of 3.43 kcal/g while T2DM induction was done by giving high-fat diet contained total energy of 5.29 kcal/g and also intraperitoneal injection of low dose streptozotocin 30 mg/kgbw. Darapladib (Glaxo Smith Kline) was given orally 20 mg/kgbw once daily according to the serial time group given.

Laboratory method

Lipid profiles measured total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol in rat blood serum

(EnzyChrom™ kit). T2DM was diagnosed by measuring fasting blood glucose level 1 week after streptozotocin injection taken from rat's tail with the result of >7.0 mmol/L (GlucODR blood glucose test meter, All Medicus Co., Ltd., Dongan-gu, Anyang-si, Korea), and insulin level in rat's blood plasma (Rat INS ELISA kit Cat. No. E-EL-R 2466). IR was calculated using homeostatic model assessment-IR (HOMA-IR) formula in rat model. HOMA-IR's result of >1.716 can be categorized as IR with sensitivity of 83.87% and specificity of 80.56% [8]:

$$\text{HOMA-IR} = \text{fasting blood glucose (mmol/L)} \times \text{fasting insulin plasma (IU/L)} / 14.1.$$

Immunofluorescence analysis

Expression of NF-κB was measured by immunofluorescence staining. Aortic tissue that previously fixed with PHEMO buffer and processed by immunofluorescence labeling with antirat antibody NF-κBp65 and HIF-1α to ensure NF-κB activation in the nucleus (BIOS Inc., Boston, MA, USA). Those parameters were observed with confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan) and quantitatively analyzed using Olympus Fluoview Software (version 1.7A; Olympus Corporation).

LPC measurement

LysoPC in serum level and aortic homogenate was measured with 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (16:0), cat 85567P, lot 16LPC-79, Avanti Polar Lipids, Inc., 700 Industrial Park Drive, Alabaster, Alabama 35007-9105, USA (6440 Triple Quadrupole LCMS/MS System, Agilent Technologies, USA). Procedure of Lyso-PC analysis referred to the Takatera's method (Blood LPC levels and characteristic molecular species in neonates: Prolonged low blood LPC levels in very low birth weight infants. *Pediatr Res*: 477-482, 2007).

IMT measurement

Slide preparation of blood vessel to measure IMT was processed using paraffin block and hematoxylin-eosin staining. IMT was measured using Dot Slide Olyvia software.

Statistical analysis

Results are expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) test used to determine the difference of control and study groups both in 8 weeks and 16 weeks serial time. It was continued by *post hoc* test using Duncan method to detect the differences in each intervention group. The level of statistical significance was defined by p<0.05 (SPSS software version 20 IBM Corporation, New York, NY, USA).

RESULT

IR analysis in darapladiib treatment

Fasting blood glucose, insulin plasma level, and IR calculation using HOMA-IR formula are shown in Fig. 1. T2DM group at 8 weeks and 16 weeks serial times has a higher level of fasting blood glucose (8.46 ± 0.91 and 7.89 ± 0.50), higher insulin plasma (3.48 ± 0.50 and 11.60 ± 1.19), and abnormal result of HOMA-IR (2.07 ± 0.16 and 6.48 ± 0.55) than normal group. Darapladiib treatment group has a normal level of fasting blood glucose (5.46 ± 0.63 and 5.44 ± 0.60), insulin plasma level (1.64 ± 0.11 and 2.43 ± 0.24), and HOMA-IR (0.64 ± 0.11 and 0.93 ± 0.08). Mann-Whitney test shows that darapladiib treatment can significantly (p<0.05) normalize fasting blood glucose, insulin plasma level, and IR in T2DM group at both 8 weeks and 16 weeks serial time.

Lyso-PC analysis

Lyso-PC in plasma level is shown in Table 1. T2DM group at 8 weeks and 16 weeks serial times has a higher level of lyso-PC (300.124.4 ± 164.787 and 118.469.4 ± 31.989.84) than normal group. Darapladiib treatment group has a lower level of lyso-PC (123.214.4 ± 32.708.87 and 87.038.6 ± 17.077.06). Mann-Whitney test show that darapladiib treatment can significantly (p<0.05) lowering lyso-PC level 8 weeks treatment [9].

Immunofluorescence analysis

Mean inflammation rate is shown in Fig. 2. T2DM group tends to have a higher level of NF-κB activation, whereas darapladiib treatment group has a lower level near to the normal one. One-way ANOVA test with *post hoc* Tukey method shows a significant value (p<0.05) for darapladiib in lowering NF-κB activation of aortic tissue at two serial time treatments (Fig. 2a). Immunofluorescence staining using double staining with FITC and rhodamine secondary antibody for NF-κB activation show qualitative expressions of specific inflammation marker at aortic tissue. Fig. 2b shows that T2DM group has maximum fluorescence intensity while normal and darapladiib group has low-medium fluorescence intensity.

Table 1: Lyso-PC level at each group

Variable	Group	8 weeks	16 weeks
Plasma Lyso PC	N	84.790.25±31.197.04 ^a	95.347.5±43.533.42 ^a
	DM	300.124.4±164.787 ^b	118.469.4±31.989.84 ^a
	DMDP	123.214.4±32.708.87 ^a	87.038.6±17.077.06 ^a

Different letter shows significant value using *post hoc* multiple comparison method (p<0.05)

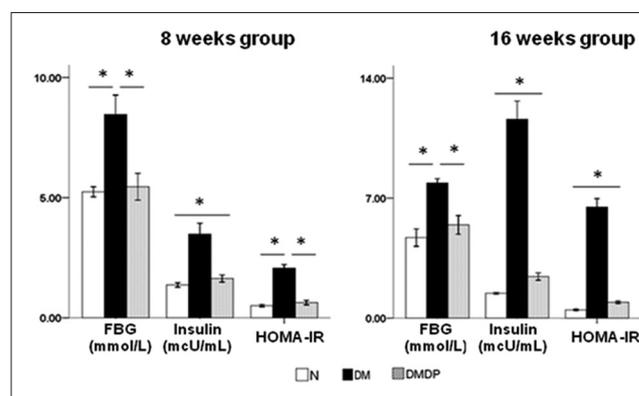


Fig. 1: Effect of darapladiib treatment on fasting blood glucose, insulin plasma, and insulin resistance level at 8 weeks and 16 weeks treatment. Mann-Whitney test showing significant result (*) at p<0.05. N: Normal group, DM: Type 2 diabetes mellitus group, DMDP: Type 2 diabetes mellitus with darapladiib treatment

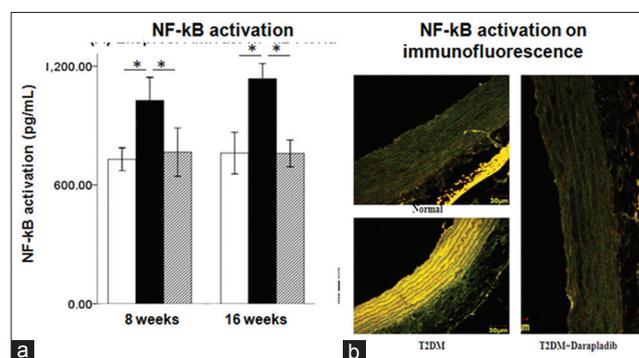


Fig. 2: Immunofluorescence results showing cytokine expression in aortic tissue (×400). N: Normal group, DM: Type 2 diabetes mellitus group, DMDP: Type 2 diabetes mellitus with darapladiib treatment. (a) Mean value of quantification result in NF-κB expression. NF-κB was significantly inhibited in darapladiib group both at 8 weeks and 16 weeks serial treatment (p=0.044 and p=0.001 vs. T2DM group), (b) double staining rhodamine and FITC secondary antibody (yellow color) show NF-κB expression in the nucleus. Normal and darapladiib group has dim intensity than T2DM group

IMT analysis

IMT measurement is shown in Table 2 and Fig. 3. T2DM group at 8 weeks and 16 weeks serial times has a higher level of IMT (92.57 ± 0.96 and 91.62 ± 0.57) than normal group. Darapladib treatment group has a lower level of IMT (87.82 ± 1.39 and 84.56 ± 1.25). Mann-Whitney test shows that darapladib treatment can significantly (p<0.05) lowering IMT level at 8 weeks and 16 weeks treatment.

DISCUSSION

T2DM was associated with increased risk of cardiovascular disease with hypothesis said IR, hyperglycemia, dyslipidemia, and inflammatory processes have an important role in every step of atherosclerotic plaque formation and rupture [10]. Inflammation plays a key role in T2DM pathogenesis and is related to the increase of cardiovascular disease risk. Hyperglycemia that occurs in T2DM stimulate the endothelial inflammation response [11].

Lipid metabolism becomes dysregulated in a variety of diseases, including obesity and diabetes [12]. Dyslipidemia that occurred in mice that were given HFD and STZ was similar to the metabolic profiles in individuals with type 2 diabetes [13]. Weight gain and the occurrence of dyslipidemia are predisposing factors of obesity that plays a major role in IR [14]. The buildup of toxic metabolites of lipids (fatty acyl CoA, diacylglycerol, and ceramide) in skeletal muscle, liver adipocytes, beta-cells, and arteries contribute to the development of IR, beta-cell dysfunction, and atherosclerosis [15]. Animal models of T2DM given a combination of a high-fat diet experienced hyperinsulinemia, IR, and/or glucose intolerance, followed by STZ as beta-cell toxin that caused degeneration of functional beta-cells. Both of these stressors are designed to resemble the pathology of type 2 diabetes in human in a shorter amount of time [16,18].

There are several mechanisms that link Lp-PLA2 with diabetes, inflammation state associated with an increase of Lp-PLA2 activity in hydrolyzing oxidized phospholipids induce IR and increase T2DM risk.

The increase in Lp-PLA2 is considered as a marker of inflammation in adipose tissue and the increase of fatty acids or adipokine flow that promote IR and pancreatic beta-cell failure [19]. Many studies associated Lp-PLA2 with cardiovascular disease but only few studies established the relationship of Lp-PLA2 activity with diabetes, especially T2DM.

NF-κB transduction signaling pathway in aortic endothelium is a process that happens in the early stage of atherosclerosis lesion formation [20]. NF-κB activation leads to the activation of several genes that play a role in the pathogenesis of the formation of atherosclerosis lesion [21,22]. NF-κB plays a crucial role in vascular physiopathology, so it participates various complication associated with DM through its role to induce systemic inflammation. NF-κB reflects inflammatory state at aortic tissue and NF-κB is correlated with impaired of blood glucose level, evidence of dyslipidemia, progression of inflammatory state, vasodilatation, and IR [23].

Inhibitor of IκB kinase (IKK) signaling pathway is one of many key mechanisms that is activated by oxidative stress and inflammation, and it regulates the cytokines that control the process of cellular apoptosis, cellular cycle progression, and inflammation [24]. NF-κB is activated by several factors that become abnormal in T2DM patients such as glucose, fatty acids, advanced glycation end-product, and toll-like receptor. NF-κB is the final pathway that connects all of the activated inputs in T2DM that eventually leads to atherosclerosis response. NF-κB regulates several molecules that plays some roles in diabetic atherosclerosis process such as tumor necrosis factor (TNF)-α, interleukin (IL-1), and IL-6 which can worsen the IR, toll-like receptors, and resistin. NF-κB regulator proteins such as inhibitor κB (IκB) and distal targets such as c-jun N-terminal kinase are associated with insulin sensitivity and atherosclerosis [23].

NF-κB activation is the process of NF-κB subunit translocation from cytoplasm to nucleus and stimulates the expression of pro-inflammatory genes such as cytokines (TNF-α and interleukin), adhesion molecules (ICAM-1 and VCAM-1), and chemokines. Activation of NF-κB initiates atherosclerosis process, and endothelial dysfunction is characterized by an increase in iNOS, platelet adhesion, and migration and proliferation of smooth muscle cells. Signs of inflammation may occur at any time, from the initial to the later stage of atherosclerosis development [25-27]. AGE may activate vascular smooth muscle cell NF-κB and mediate the migration and proliferation of smooth muscle cells [28].

In this study, there was an increase of lyso-PC, NF-κB, and IMT in T2DM rat model. Increased expression of NF-κB because of cytokines, high glucose levels, and AGE explains the occurrence of vascular dysfunction in diabetes, and the activation of NF-κB can enhance the activation of important genes involved in the pathology of atherosclerosis [22].

Lp-PLA2 appeared as an effective marker in detecting cardiovascular disease. Macrophages that infiltrate adipose tissue increase the production of Lp-PLA2. Lp-PLA2 induces the expression of several pro-inflammatory cytokines through the accumulation of its product in the form of lyso-PC. Lp-PLA2 activity is also associated with oxidative stress events through its activities in modifying phospholipids. Free radicals under hyperglycemia conditions play a role in increasing lipoprotein oxidation process; thereby increasing Lp-PLA2 substrates. In another word, Lp-PLA2 helps increasing the severity of inflammation, oxidative stress, and microvascular dysfunction mediating the pathogenesis of cardiovascular disease, especially atherosclerosis in T2DM [29].

NF-κB and MAPK pathways are closely associated with inflammatory responses in cells. NF-κB and MAPK pathways were related to the regulation of Lp-PLA2. NF-κB signaling pathway could interfere with insulin signaling through an inhibition of insulin receptor substrate 1, Akt (protein kinase B), and eNOS phosphorylation, and lead to a decrease in NO production, greater incidence of inflammation, and endothelial dysfunction [30].

Table 2: IMT in each group

Variable	Group	8 weeks	16 weeks
IMT (µm)	N	87.8363±10.9843 ^a	87.8363±10.9843 ^a
	DM	92.5689±0.96093 ^b	91.6243±0.56537 ^b
	DMDP	87.821±1.39084 ^a	84.562±1.25315 ^a

Different letter shows significant value using *post hoc* multiple comparison method (p<0.05)

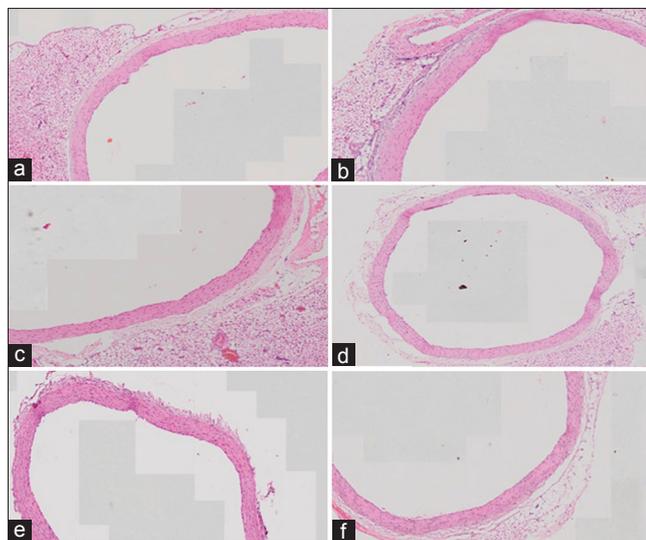


Fig. 3: Intima-media thickness in each intervention group (a) N8, (b) N16, (c) DM8, (d) DM16, (e) DMDP 8, (f) DMDP 16

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Therapeutic strategies using antibodies or molecules that target IKK-b-NFkB (salicylates, salsalate), TNF- α (etanercept, infliximab, and adalimumab), IL-1 β (anakinra), and IL-6 (tocilizumab) to cure the inflammation may improve insulin sensitivity and beta-cell function [31]. This study proved that the effect of darapladib as a selective inhibitor of Lp-PLA2 in reducing Lyso-PC level, aortic NF-kB expression, IMT, and also affect insulin sensitivity in type 2 diabetes Sprague-Dawley rats models.

CONCLUSION

This study proves the role of darapladib as anti-inflammatory and antiatherogenesis properties to diminish lyso-PC and NF-kB, lowering IMT and improve IR in T2DM rat model.

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