

Lp-Pla2 Selective Inhibitor (Darapladib) Effect in Lowering Insulin Resistance and Aortic tissue Inflammation at type 2 Diabetes Mellitus

Titin Andri Wihastuti¹, Dinda Zahra Putri Andiyani¹, Sri Andarini¹, Teuku Heriansyah^{2*}

¹Department of Biomedicine, Faculty of Medicine, Brawijaya University, Malang, Indonesia.

²Department of Cardiology and Vascular Medicine, Faculty of Medicine, Syiah Kuala University, Banda Aceh, Indonesia.

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ABSTRACT

Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is an enzyme with several pro-inflammatory properties that involved in the pathogenesis of atherosclerosis but some investigation shows controversial views regarding its biological role. We examined the effect of a selective inhibitor of Lp-PLA₂ (darapladib) to the inflammation marker such as nuclear factor kappa B (NF-κB) and interleukin-6 (IL-6) expression in aortic tissue and insulin resistance of type 2 diabetes mellitus (T2DM) rat model. 30 Sprague-dawley rats were randomly divided into the 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 insulin resistance in mice. Eight weeks and sixteen weeks after treatment, we compared expression of NF-κB and IL-6 in aortic tissue. Darapladib treatment group exhibited significant reduction of insulin resistance (0.64±0.11 vs 2.07±0.16, p<0.05 at 8 weeks; and 0.93±0.08 vs 6.48±0.55 at 16 weeks) compared with T2DM group. On immunofluorescence analysis, darapladib significantly decreased NF-κB and IL-6 expression at 2 serial treatments. These data suggested that Lp-PLA₂ played a role in inflammation process and improve insulin resistance occurring in the metabolic disorder.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is one of the most non-communicable diseases involving multiple genes and environmental factors that characterized by impaired insulin secretion through a dysfunction of pancreatic β-cells and insulin resistance (Ozougwu *et al.*, 2013). It is associated with an increased risk of atherosclerotic cardiovascular disease (Niskanen *et al.*, 1998). Prolonged exposure to hyperglycemia altered vascular homeostasis and associated with vascular inflammation, that has been hypothesized underlying atherosclerosis process in T2DM condition (Kim *et al.*, 2010).

Both free fatty acid and AGE directly activate nuclear factor-κB (NF-κB) that required for the transcription of most pro-inflammatory molecules, adhesion molecules, cytokine in the pathology of atherosclerosis and diabetic complication (Patel and Santani, 2009). Study showed that interleukin-6 (IL-6) level are used to predict risk of cardiovascular disease in the development of T2DM because it had a role in the pathogenesis of insulin resistance (Hameed *et al.*, 2015).

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 the 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 and oxidized fatty acid. Lp-PLA₂'s pathways that related to inflammation has been hypothesized to have a role in atherosclerosis process.

* Corresponding Author

Teuku Heriansyah, Department of Cardiology and Vascular Medicine,
Faculty of Medicine, Syiah Kuala University, Banda Aceh
Faculty of Medicine, Syiah Kuala University, St. Teuku Nyak Arief
Darussalam, Banda Aceh, Aceh, Indonesia.
Phone: 6281-1661-8282, Email: teuku_hery@unsyah.ac.id

Darapladib is a reversible Lp-PLA₂ inhibitor that has been extensively tested in vitro and in vivo (Stafforini and Zimmerman, 2014). However, studies show controversial results about Lp-PLA₂'s role in cardiovascular disease (Hassan, 2015). So, this study aims to determine the role of Lp-PLA₂ selective inhibitor to the expression of inflammation marker such as NF-κB and IL-6 in the T2DM rat model.

MATERIAL AND 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 on 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 400/EC/KEPK/10/2016 at October, 13th 2016.

Animals

Acclimatization phase

Thirty male, six-week-old, Sprague-Dawley rats (Bogor Agricultural University, Indonesia), 150-200 gram weight each experienced acclimatization phase during two weeks adjustment period. All animals were given normal diet consist of comfeed PARS, wheat flour and water as much as 26 gram/rats/day. The rest amount of feed was taken and weighed every 24 hours. After the acclimatization phase, then rats continued to the randomization process to be divide into treatment phase.

Treatment phase

In this phase, Sprague-dawley rats were divided into the negative control (normal) group, positive control group (T2DM) and study group (T2DM with darapladib treatment). Each group was divided into 2 serial treatments: 8 weeks and 16 weeks. The negative control group was given normal rats food contained the total energy of 3.43 kcal/g while T2DM induction was done by giving high-fat diet contained the total energy of 5.29 kcal/g and also intraperitoneal injection of low dose streptozotocin 30mg/kg bw. Darapladib (Glaxo Smith Kline) was given orally 20 mg/kg bw once daily according to the serial time group given.

Laboratory Method

Lipid profiles measured total cholesterol, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol in rat blood serum (EnzyChrom™ kit). T2DM was diagnosed by measuring fasting blood glucose level one week after streptozotocin injection taken from rat's tail with the result of >7.0mmol/L (GlucodR blood glucose test-meter All Medicus Co.Ltd, Dongan-gu, Anyang-si, Korea). The insulin level in rat's blood plasma (Rat INS ELISA kit Cat. No. E-EL-R 2466). Insulin resistance was calculated by using homeostatic model assessment-insulin resistance (HOMA-IR) formula in rat model. HOMA IR's result of >1.716 can be categorized as insulin resistance with a sensitivity of 83.87% and specificity of 80.56%):

$HOMA-IR = \text{fasting blood glucose (mmol/L)} \times \text{fasting insulin plasma (IU/L)} / 14.1$ (VanDjik, 2013)

Immunofluorescence Analysis

Expression of NF-κB and IL-6 were measured by immunofluorescence staining. Aortic tissue that previously fixed with PHEMO buffer and processed by immunofluorescence labeling with anti-rat antibody IL-6 and fluorescein isothiocyanate (FITC) secondary antibody; and anti-rat 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).

Statistical Analysis

Results are expressed as the mean ± SD. One-way analysis of variance (ANOVA) test used to determine the difference between the control and the 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

Insulin resistance analysis in darapladib treatment

Fasting blood glucose, insulin plasma level, and insulin resistance calculation using HOMA-IR formula are shown in figure 1. T2DM group at 8 weeks and 16 weeks serial times have 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 the abnormal result of HOMA-IR (2.07 ± 0.16 and 6.48 ± 0.55) than normal group. Darapladib treatment group have 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 darapladib treatment can significantly ($p < 0.05$) normalize fasting blood glucose, insulin plasma level and insulin resistance in the T2DM group at both 8 weeks and 16 weeks serial time.

Lipid profile analysis in darapladib treatment

Lipid profile result are shown in figure 2. T2DM group at 8 weeks and 16 weeks serial times tend to have a higher level of total cholesterol (123.00 ± 2.86 and 111.72 ± 7.30), higher level of LDL-cholesterol (95.53 ± 8.66 and 88.24 ± 6.23), and a lower level of HDL-cholesterol (4.96 ± 0.41 and 13.96 ± 0.87) than normal group. Mann-Whitney test show that darapladib treatment at 8 weeks and 16 weeks serial time can significantly ($p < 0.05$) normalize total cholesterol (97.96 ± 1.70 and 98.96 ± 3.24), LDL-cholesterol (85.92 ± 6.84 and 61.52 ± 6.03) and HDL-cholesterol (16.44 ± 1.24 and 20.80 ± 2.76) near to the normal group.

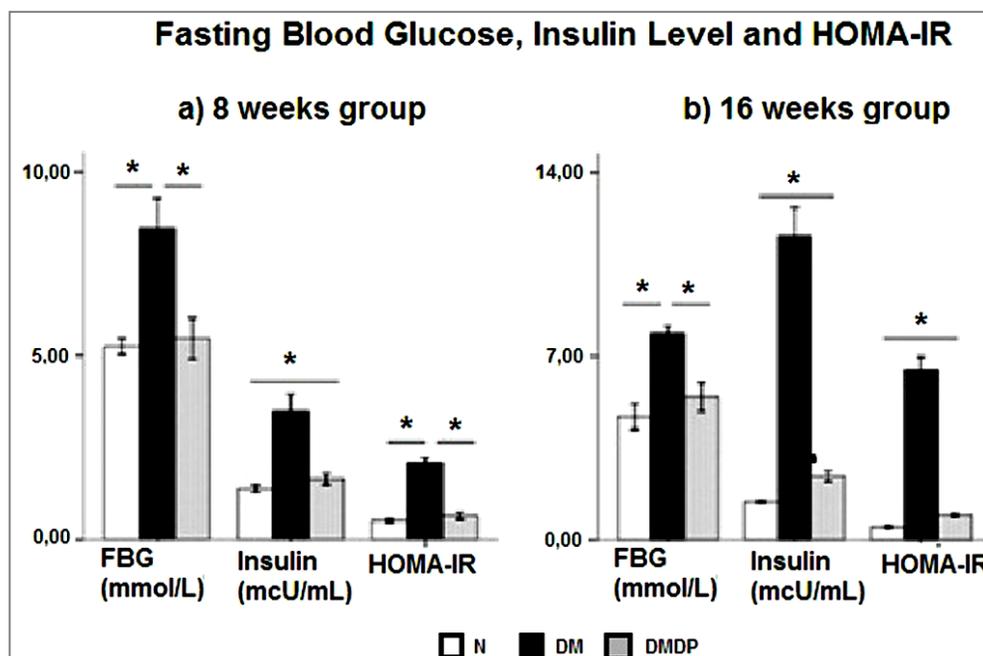


Fig. 1: Effect of darapladi treatment on fasting blood glucose, insulin plasma and insulin resistance level at 8 weeks and 16 weeks treatment. Mann whitney test showing significant result (*) at $p < 0.05$. N: normal group, DM: Type 2 diabetes mellitus group, DMDP: type 2 diabetes mellitus with darapladi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

Lipid Profile

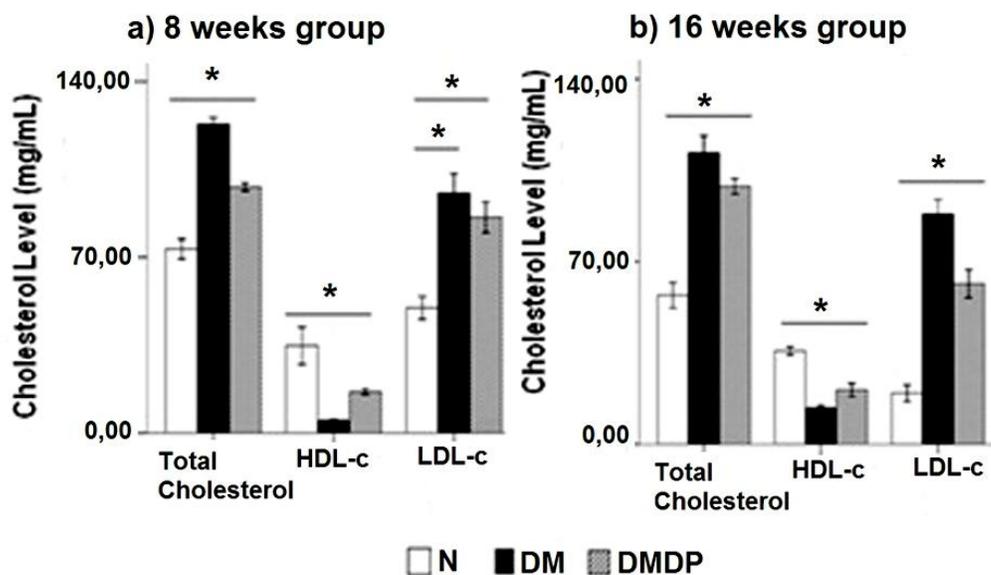


Fig. 2: Effect of darapladi treatment on total cholesterol, HDL cholesterol and LDL cholesterol level at 8 weeks and 16 weeks treatment. Mann whitney test showing significant result (*) at $p < 0.05$. N: normal group, DM: Type 2 diabetes mellitus group, DMDP: type 2 diabetes mellitus with darapladi treatment.

Immunofluorescence analysis

Mean inflammation rate are shown in figure 3. T2DM group tends to have a higher level of NF- κ B and IL-6 expression whereas darapladi treatment group have 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 darapladi in lowering NF- κ B & IL-6 expression of aortic tissue at 2 serial time treatments

(figure 3a and 3c). Immunofluorescence staining using rhodamine secondary antibody for IL-6 expression and double staining with FITC and rhodamine secondary antibody for NF- κ B activation show qualitative expressions of specific inflammation marker at the aortic tissue. Figure (3b) and (3d) show that T2DM group has maximum fluorescence intensity while normal and darapladi group has low-medium fluorescence intensity.

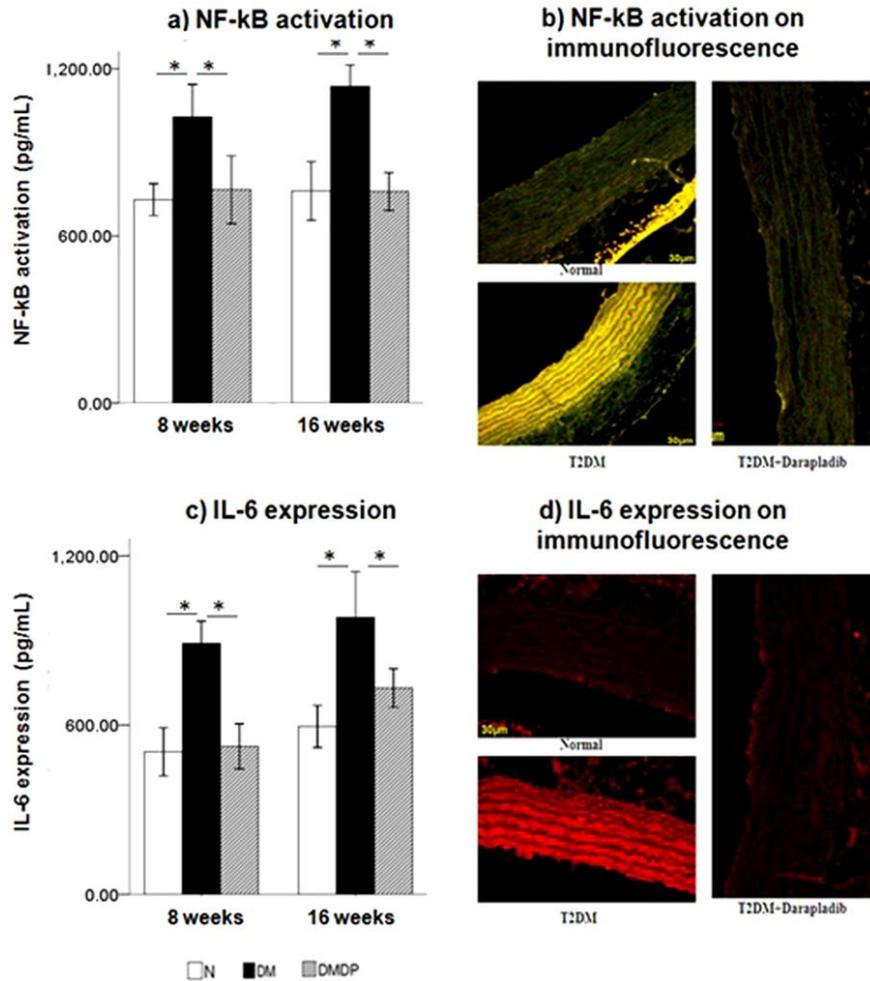


Fig. 3: Immunofluorescence results showing cytokine expression in aortic tissue (400x). N: normal group, DM: Type 2 diabetes mellitus group, DMDP: type 2 diabetes mellitus with darapladib treatment. (A) Mean value of quantification result in NF-κB expression. NF-κB was significantly inhibited in darapladib 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 nuclues. Normal and darapladib group has dim intensity than T2DM group; (C) Mean value of quantification results in IL-6 expression. IL-6 was significantly inhibited in darapladib group both at 8 weeks and 16 weeks serial treatment ($p=0.025$ and $p=0.001$ vs T2DM group); (D) Rhodamine secondary antibody (red color) show IL-6 expression. Normal and darapladib group has dim intensity than T2DM group

DISCUSSION

Lipid Profile and Insulin Resistance in T2DM group and Darapladib Treatment Group

Type 2 diabetes mellitus (T2DM) was associated with increased risk of cardiovascular disease with hypothesis said insulin resistance, hyperglycemia, dyslipidemia and inflammatory processes have an important role in every step of atherosclerotic plaque formation and rupture (Ziegler, 2005). Inflammation plays a key role in T2DM pathogenesis and is related to the increase in cardiovascular disease risk. Hyperglycemia and dyslipidemia that occur in T2DM stimulate the endothelial inflammation response (Mazzone *et al.*, 2008).

Lipid metabolism becomes dysregulated in a variety of diseases, including obesity and diabetes. Dyslipidemia that occurred in mice that were given HFD and STZ was similar to the metabolic profiles in individuals with type 2 diabetes (Skovso, 2014). Weight gain and the occurrence of dyslipidemia are

predisposing factors of obesity that plays a major role in insulin resistance (Shaw *et al.*, 2010). 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 insulin resistance, beta cell dysfunction and atherosclerosis (Reaven, 2005). Animal models of type 2 diabetes mellitus given a combination of a high-fat diet experienced hyperinsulinemia, insulin resistance 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 (Zhang *et al.*, 2008).

Administration of darapladib in T2DM group had significantly different parameters compared to the diabetic group. There was an improvement in lipid profile values and insulin resistance with darapladib treatment. 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 insulin resistance and increase type 2 diabetes mellitus 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 insulin resistance and pancreatic beta cell failure (Nelson *et al.*, 2012). Many studies associated Lp-PLA2 with cardiovascular disease, but only a few studies established the relationship of Lp-PLA2 activity with diabetes, especially type 2 diabetes mellitus.

Inflammation Marker in T2DM group and Darapladi Treatment Group

NF- κ B transduction signaling pathway in aortic endothelium is a process that happens in the early stage of atherosclerosis lesion formation (Hajra *et al.*, 2000). NF- κ B activation leads to the activation of several genes that play a role in the pathogenesis of the formation of atherosclerosis lesion (Patel and Santani, 2009). NF- κ B plays a crucial role in vascular physiopathology so it participates various complication associated with DM through its role to induce systemic inflammation. NF- κ B reflects inflammatory state at the aortic tissue and NF- κ B is correlated with impaired of blood glucose level, evidence of dyslipidemia, the progression of an inflammatory state, vasodilatation and insulin resistance (Indira and Ahilash, 2013).

Endothelial dysfunction is the first phenomenon happened in line with insulin resistance that contributes to the pathogenesis of cardiovascular disease, which interleukin-6 (IL-6) is proinflammatory cytokine correlates with those abnormalities (Esteve *et al.*, 2007). Studies show that circulating IL-6 was directly related to endothelial dysfunction and insulin resistance, that can be some kind of predictor to the development of T2DM and cardiovascular events (Pradhan *et al.*, 2001; Shinohara *et al.*, 2012). Prolonged tissue injury because of hyperglycemia state increase inflammatory cytokine, such as TNF- α and IL-6 that activate inflammatory cascade mediated by cellular adhesion molecules in endothelial cells called endothelial activation (Antonaides *et al.*, 2004). Anti-inflammatory substances are given to diabetics may restore glucose level and diminish risk of cardiovascular events (Tabit *et al.*, 2010).

Inhibitor of I κ B 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 (Tilstra *et al.*, 2011). NF- κ B is activated by several factors that become abnormal in type 2 diabetes mellitus patients, such as glucose, fatty acids, advanced glycation end product, and toll-like receptor. NF- κ B is the final pathway that connects all of the activated inputs in type 2 diabetes mellitus that eventually leads to atherosclerosis response. NF- κ B regulates several molecules that plays some roles in diabetic atherosclerosis processes, such as TNF- α , IL-1 and IL-6 which can worsen the insulin resistance, TLR and resistin.

NF- κ B regulator proteins such as inhibitor κ B (I κ B) and distal targets such as c-jun N-terminal kinase (JNK) are associated

with insulin sensitivity and atherosclerosis (Indira *et al.*, 2013). NF- κ B activation is the process of NF- κ B subunit translocation from cytoplasm to nucleus and stimulates the expression of proinflammatory genes such as cytokines (TNF- α and interleukin), adhesion molecules (ICAM-1 and VCAM-1) and chemokines (Wihastuti *et al.*, 2016). Activation of NF- κ B initiates atherosclerosis process and endothelial dysfunction is characterized by an increase in iNOS, platelet adhesion, and migration and proliferation of smooth muscle cells (Wihastuti *et al.*, 2014). Signs of inflammation may occur at any time, from the initial to the later stage of atherosclerosis development (Heriansyah *et al.*, 2016). AGE may activate vascular smooth muscle cell NF- κ B and mediate the migration and proliferation of smooth muscle cells (Tabit *et al.*, 2010).

In this study, there was an increase of NF- κ B and IL-6 expression in type 2 diabetes mellitus rat aorta. Increased expression of NF- κ B because of cytokines, high glucose levels, and AGE explains the occurrence of vascular dysfunction in diabetes, and the activation of NF- κ B can enhance the activation of important genes involved in the pathology of atherosclerosis (Patel and Santani, 2009).

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 lysoPC. 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 to increase the severity of inflammation, oxidative stress, and microvascular-dysfunction mediating the pathogenesis of cardiovascular disease, especially atherosclerosis in type 2 diabetes mellitus (Zhu, 2016).

NF- κ B and MAPK pathways are closely associated with inflammatory responses in cells. NF- κ B and MAPK pathways were related to the regulation of Lp-PLA2. NF- κ B 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 (Han *et al.*, 2015).

Therapeutic strategies using antibodies or molecules that target IKK- β -NF κ B (salicylates, salsalate), TNF- α (etanercept, infliximab, adalimumab), IL-1 β (anakinra) and IL-6 (tocilizumab) to cure the inflammation may improve insulin sensitivity and beta cell function (Esser *et al.*, 2015). This study proved that the effect of darapladi as a selective inhibitor of Lp-PLA2 in reducing aortic NF- κ B and IL6 expression may affect insulin sensitivity; this was proved by the occurrences of normoglycaemia, normoinsulinemia, and normal HOMA-IR level and lipid profile in the treatment group of type 2 diabetes Sprague Dawley rats models.

CONCLUSION

This study proves the role of darapladib as anti-inflammatory properties to diminish NF- κ B and IL-6 levels and improve insulin resistance in the T2DM rat model.

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