Research Article

The effect of Darapladib as a lipoprotein-associated phospholipase A2 inhibitor to interleukin-1β and interleukin-6 on diabetes mellitus Sprague-Dawley rat model: A new therapeutic target of diabetes mellitus to decrease cardiovascular risk

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ABSTRACT

Background: Death related to cardiovascular events occurs due to atherosclerotic mainly based on plaque disruption. Inflammation is the most important mechanism of it. Interleukin-1β (IL-1β) and IL-6 has already known as inflammatory markers. Recent study reports that lipoprotein-associated phospholipase A2 (Lp-PLA2) regulates the inflammatory process. This study wanted to investigate whether Darapladib as Lp-PLA2 inhibitor can prevent the inflammatory process in type 2 diabetes mellitus (T2DM) rat model by reducing pro-inflammatory cytokine, especially IL-1β and IL-6. **Methods:** A total of 30 Sprague-Dawley rats were divided into three groups which were normal, T2DM, and T2DM with Darapladib administration (DMDP) group. Each group was divided into 8 and 16 weeks (early and late phase). The parameters in this study are IL-1β and IL-6 expressions measured using immunofluorescence. **Results:** In the early phase, the IL-1β expression in normal group was significantly different from DM, while in DMDP group showed a decrease trend although not significantly differ from the DM. The IL-6 expression was significantly different in normal and DM group, and also in DMPDP compared with DM group. In the late phase, the IL-1β expression in normal group was significantly different from the DM group, while in DMDP group showed a decrease trend although not significantly differ from the DM group. The IL-6 expression in normal group was significantly different from the DM group, while in DMDP group showed a decrease trend but not significantly differ from the DM group. **Conclusion:** Inflammatory process in T2DM Sprague-Dawley rats model could be reduced as the effect of inhibiting Lp-PLA2 activity.

KEY WORDS: Atherosclerotic, Inflammation, Interleukin-1β, Interleukin-6, Lipoprotein-associated phospholipase A2, Lipoprotein-associated phospholipase A2 inhibitor

INTRODUCTION

Cardiovascular disease currently listed as the main cause of death in the world. Ischemic heart disease and stroke are on the top list of 10 causes death worldwide and have remained the leading causes of death globally in the past 15 years.[1,2] A great majority of death related to cardiovascular events occur due to atherosclerotic and its acute complications, mainly based on plaque disruption. Inflammation is the most important mechanism of plaque disruption, and it is important to understand its molecular mechanism.[3-5]

Inflammatory processes are now recognized to play a central role in the pathogenesis of atherosclerosis and its complications. Plasma levels of several inflammation markers have been found to be associated with future cardiovascular risk. These markers include cell adhesion molecules, cytokines, proatherogenic enzymes, and C-reactive protein (CRP).[6]

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Interleukin-1 (IL-1) is an apical proinflammatory mediator in acute and chronic inflammation.[7] Two related genes (IL-1α and IL-1β) code for two different proteins that bind the same IL-1 receptor (type 1). IL-1α is synthesized as a fully active peptide that remains membrane-bound or may be released from the cytoplasm during cell death. IL-1α thereby participates more prominently in local response to injury and less in the systemic inflammatory response.[7,8] IL-1β is the main form of circulating IL-1 and is initially synthesized as a precursor (proIL-1β) that becomes activated by caspase-1 cleavage in the setting of a macromolecular structure known as the inflammasome.[7,9] Activation of inflammasome following tissue injury induces a local surge of IL-1β that significantly amplifies the inflammatory response, recruiting more inflammatory cells, stimulating metalloproteinase activities, and ultimately inducing inflammatory cell death (pyroptosis) in leukocytes and resident cells.[10]

IL-6 is known to be an inflammatory marker. An increased serum level of IL-6 associated with an increased risk of cardiovascular disease.[11-13] Elevated levels of IL-6 are also involved in the pathogenesis of insulin resistance. T2DM is a proinflammatory condition and increases the risk of coronary heart disease.[14-16]

Lipoprotein-associated phospholipase A2 (Lp-PLA2) known as platelet activating factor acetylhydrolase (PAF-AH), its enzyme composes of 45 KDa molecular, secreted by monocyte, T-lymphocyte, macrophages, mast cells, live cells, mostly by inflammatory cells. Lp-PLA2 hydrolyzes fatty acids at sn-2 position into two polyunsaturated fatty acid and lysophospholipids which as marker of Lp-PLA2 activity, and specifically catalyzes the hydrolysis of oxidized low-density lipoprotein (ox-LDL) into lysophosphatidylcholine (LysoPC) and oxidized fatty acid.[17] LysoPC induces proinflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-α (TNF-α).[18] Recent study reports that Lp-PLA2 regulates the proinflammatory and anti-inflammatory process.[19] Inflammatory processes have been increasingly recognized as important components of the pathogenesis of metabolic syndrome,[20] and Lp-PLA2 has been reported to correlate with several components of metabolic syndrome.[21,22]

Therefore, this study wanted to find out whether Darapladib as the new therapeutic target as Lp-PLA2 inhibitor can prevent the inflammatory process in T2DM by reducing pro-inflammatory cytokine, especially IL-1β, and IL-6.

MATERIALS AND METHODS

This research was conducted at the Central Laboratory of Biological Sciences, Brawijaya University. Ethical approval was obtained for the animal treatment and experimental processes in this study from the Animal Care and Use Committee Brawijaya University Number 400/EC/KEPK/10/2016.

Study Design

The animal model used in this experiment is using the combination of low doses of streptozotozin (STZ) and high-fat diet, which could induce metabolic syndrome mimicking human criteria.[23] This study used Sprague-Dawley rat (4 weeks old, weighted around 150–200 g). Samples were obtained from Bogor Agricultural University, Bogor, Indonesia. These rats were divided into three main groups: Normal, Type 2 diabetes mellitus (T2DM), and T2DM with Darapladib administration (DMDP) groups. Each group consists of 2 serial treatment time: 8 and 16 weeks. T2DM model group was fed with high-fat diet and injected with STZ intraperitoneal low dose 35 mg/kg BW. Darapladib was purchased from GlaxoSmithKline. It was administered to the animal models daily as much as 20 mg/kg of rat's body weight. The control groups were administered a placebo. Normal rat food contained 3.43 kcal/g total energy calories, while the high-fat diet contained 5.29 kcal/g total calorie energy. 30 g of food were given for each rat every day. To ensure the T2DM model, we measured blood glucose, homeostatic model assessment-insulin resistance (HOMA-IR), and lipid profiles. The result can be seen in our previous research.[24]

Il-1β and Il-6 Expression Measurement Using Immunofluorescence

Il-1β and IL-6 in heart were measured by immunofluorescence. The heart tissue was previously fixed with PHEMO buffer (68 mM PIPES, 25 mM, HEPES, pH 6.9, 15 mMEGTA, 3 mM $MgCl_2$, 10% [v/v] dimethyl sulfoxide containing 3.7% formaldehyde, and 0.05% glutaraldehyde) and was processed by immunofluorescence labeling with anti-rat antibody IL-1β using rhodamin secondary antibody (BIOS Inc. Boston, M.A, USA) and anti-rat antibody IL-6 using FITC secondary antibody (Santacruz, USA). This parameter was observed with confocal laser scanning microscopy (Olympus Corp., Tokyo, Japan) and was quantitatively analyzed using Olympus FluoView Software (version 1.7A; Olympus Corporation).

Statistical Analysis

Blood glucose, insulin resistance, lipid profile, IL-1β and IL-6 expression were documented, and normality test was performed to determine the normality of the data. Data were analyzed using one-way ANOVA to determine the effect of time serial factor, Darapladib administration, and the interaction between time and Darapladib administration on the expression of IL-1β and IL-6 in Sprague-Dawley rats' heart. This analysis

was continued by *post hoc* using Duncan method to detect parameter differences in each treatment group. All of the analyzes were performed using SPSS statistical software, version 21 and $P < 0.05$ was considered to be statistically significant.

RESULTS

To ensure that the rats were developing T2DM, we measured Fasting blood glucose, insulin resistance, and lipid profile. The result can be seen in our previous publication.[24] T2DM model was made successfully if fasting blood glucose >126 mg/dl and insulin resistance (using HOMA-IR) results >1.716. Result showed that there is a significant different in lipid profile (cholesterol total, high-density lipoprotein, and LDL) between normal and DM group. This result shows that Sprague-Dawley T2DM model was successfully made.[24]

Based on Shapiro-Wilk normality test, we have the significance value of all of the groups are above 0.05 ($\alpha > 0.05$), so we can conclude that all of the data are normally distributed.

A one-way between subject ANOVA was conducted to compare the effect of Darapladib as an Lp-PLA2 inhibitor on IL-1β and IL-6 in normal group, DM group, and DMDP group in two serial time: 8 weeks and 16 weeks.

For the 8 weeks serial time, there was a significant effect of Darapladib on IL-1βexpressions at the $P < 0.05$ for the three condition ($F[2,12] = 18.45$, $P = 0.000$); and also significant effect of Darapladib administration on IL-6 expressions for the three groups ($F [2,12] = 12.28$, $P = 0.001$). Because we have found a statistically significant result in this test, we needed to compute a *post hoc* test. *Post hoc* comparison using Duncan method indicated that the mean score of IL-1β expressions in normal group (M = 542.35, standard deviation [SD] = 28.92) was significantly different than the IL-1β expressions in DM group (M = 859.93, SD = 133.62). However, the IL-1β expressions on DMDP group (M = 780.51, SD = 59.28) did not significantly differ from the DM group, although there was a decrease trend on the IL-1β expressions of the DMDP group when compared to the DM group.

Meanwhile the mean score of IL-6 expressions in normal group (M = 549.50, SD = 28.92) was significantly different from the IL-6 expressions in DM group (M = 1237.91, SD = 372.06), and the IL-6 expressions of the DMDP group (M = 867.83, SD = 76.18) were also significantly different from the DM group.

For the 16 weeks serial time, there was a significant effect of Darapladib on IL-1β expressions at the $P < 0.05$ for the three condition ($F [2,12] = 8.47$, $P = 0.005$); and also significant effect of Darapladib administration on IL-6 expressions for the three groups ($F [2,12] = 7.03$, $P = 0.010$). *Post hoc* comparison using Duncan test indicated that the mean score of IL-1β expressions in normal group (M = 838.71, SD = 69.35) was significantly different than the IL-1β expressions in DM group (M = 1373.74, SD = 197.96). However, the IL-1β expressions on DMDP group (M = 1173.86, SD = 292.44) did not significantly differ from the DM group, but there was a decrease trend of the IL-1β expressions of the DMDP group if we compared it with the DM group.

Meanwhile the mean score of IL-6 expressions in normal group (M = 946.52, SD = 61.79) was significantly different from the IL-6 expressions in DM group (M = 1627.52, SD = 243.84). However, the IL-6 expressions of the DMDP group (M = 1406.52, SD = 440.62) did not significantly differ from the DM group, though there was a decrease trend when we compare the IL-6 expressions of DMDP group with the DM group [Figure 1].

DISCUSSION

Darapladib is a selective inhibitor that targets the active-site serine residue of the enzyme Lp-PLA2 and is at present the only selective Lp-PLA2 inhibitor in Phase III clinical development.[25] The enzyme Lp-PLA2 is a 45-kDa protein with 441 amino acids and is a calcium-independent hydrolase that targets water-soluble polar phospholipids with oxidatively truncated shortened sn-2 chain.[26] It was initially described as PAF acetylhydrolase and belongs to the superfamily of structurally diverse phospholipase A2 enzymes.[27] It is secreted by several inflammatory cells that are known to play critical roles in atherogenesis (monocytes/macrophages, T-lymphocytes, and mast cells).[28] Population studies have revealed an association between Lp-PLA2 and cardiovascular events and many observations indicate that Lp-PLA2 may play a causative role in promoting plaque instability and have formed the basis for the development of inhibitors of Lp-PLA2 as a potential vascular specific atherosclerotic drug targeting inflammation.[25]

IL-1 is the first IL and affects virtually all cells and organs. It is major pathogenic mediators of inflammatory and immune disease.[29] Diabetes is a proinflammatory state with increased levels of circulating cytokine suggesting a causal role for inflammation in its etiology.[30] Chronic inflammatory process underlies the failure of the beta-cell to secrete a sufficient amount of insulin in patients with T2DM. The discrete insulitis is due to a pathological activation of the innate inflammatory/immune system by metabolic stress and is governed by IL-1 signaling.[31] This

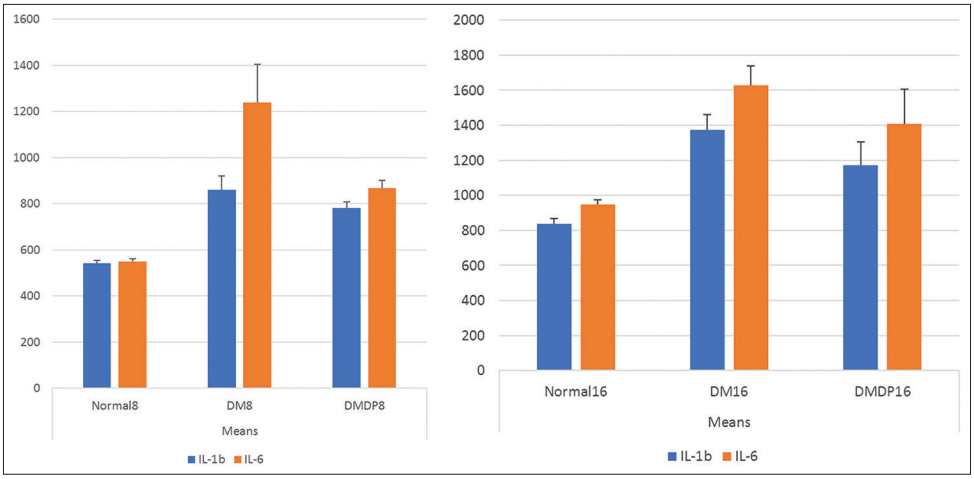

Figure 1: Darapladib could lower the interleukin (IL)-1β and IL-6 expression in Type 2 diabetes mellitus (T2DM) Sprague-Dawley rat. Left chart - 8 weeks serial time the mean score of IL-1β expressions in normal group was significantly different than the IL-1β expressions in DM group and the IL-1β expressions on T2DM with Darapladib administration (DMDP) group did not significantly differ from the DM group, although there was a decrease trend on the IL-1β expressions of the DMDP group. The mean score of IL-6 expressions in normal group was significantly different from the IL-6 expressions in DM group, and the IL-6 expressions of the DMDP group were also significantly different from the DM group. Right chart - the mean score of IL-1β expressions in normal group was significantly different than the IL-1β expressions in DM group and the IL-1β expressions on DMDP group did not significantly differ from the DM group, but there was a decrease trend of the IL-1β expressions of the DMDP group. The mean score of IL-6 expressions in normal group was significantly different from the IL-6 expressions in DM group. The IL-6 expressions of the DMDP group did not significantly differ from the DM group, but there was a decrease trend

study shows that IL-1β, a proinflammatory cytokine is significantly elevated in DM Sprague-Dawley rats model compared with the normal Sprague-Dawley rats in both serial times, in the acute phase (8 weeks) and chronic phase (16 weeks). This result is similar with the study conducted by Dinarello (2010) stated that overnutrition is the main cause of type 2 diabetes, thus exposure of human islets to glucose, leptin, or free fatty acids induces the production and release of IL-1β.[31] T2DM is a condition of systemic oxidative stress that has a major role in the pathogenesis of atherosclerosis,[32] the increase of oxidative stress on arterial walls contributes to atherogenesis.[33]

Proinflammatory cytokines including IL-1β, IL-6, and TNF-α are released from macrophages within the vessel wall during an inflammatory response. These cytokines mediate distant inflammatory effects, including activation of hepatic genes encoding acute phase reactant fibrinogen, CRP, and serum amyloid A. CRP induces synthesis of cytokines, cell adhesion molecules, and tissue factor in monocytes and endothelial cells. Tissue factor activates the extrinsic coagulation cascade, providing a link between inflammation and thrombosis.[20] Atherosclerosis has been declared as one of the inflammatory diseases since inflammatory processes play a dominant role in the various stages of the development of atherosclerosis. Those processes include endothelial dysfunction which produces proinflammatory mediators that stimulate monocyte differentiation process into a macrophage. Then macrophage developed into foam cells as the ox-LDL uptake process and local inflammatory response released.[32] Darapladib has been shown to reduce the content of many genes related with macrophage and T lymphocytes function, with a decrease in plaque and necrotic areas.[34] Inhibition of Lp-PLA2 expression by Darapladib was also found in the research conducted by Heriansyah, *et al.* (2016), stated that Darapladib can reduce oxidative stress and inflammation in atherogenesis.[35] In human atherosclerotic plaques, the expression of IL-1α and IL-1β appears to correlate with the progression of atherosclerotic plaques, with minimal expression in healthy coronary arteries, increased expression in healthy coronary arteries, increased expression in simple atherosclerotic plaques, and high expression in complicated plaques.[3,36] In atherosclerosis and atherothrombosis, the degree to which plaque is formed, progress and rupture are dependent, at least in part, on the degree of inflammation in the plaque. In human atherosclerotic plaques, the expression of IL-1α and IL-1β appears to correlate with the progression of atherosclerotic plaques.[3,36] An increase in IL-1 activity causes a destabilization of the plaque, rupture and superimposed thrombus formation.[37] In this study, although the expression of IL-1β is not significantly different in T2DM Sprague-Dawley rats model if compared with rats treated with Darapladib, but there was a decrease trend found in both serial times (8 and 16 weeks of treatment), showing that Darapladib administration in T2DM Sprague-Dawley rats model tend to lower

the IL-1β expression, and thus can lower the risk of inflammation and the formation of atherosclerotic plaque in DM.

IL-6 has been emphasized by reports of elevated circulating as well as intracardiac IL-6 levels in patients with cardiovascular diseases.[37] Increasing levels of IL-1Ra and of IL-6 as a secondary downstream mediator of IL-1β predicted adverse outcome in patients with unstable angina.[38] The injection of recombinant IL-6 increased lesion size in the aorta of apoE$^{-/-}$ and C57BI/6 mice fed with a high-fat diet and increased the expression of tissue and circulating proinflammatory cytokines (IL-1β and TNF-a).[39]

This study shows that IL-6 is significantly elevated-in both 8 weeks and 16 weeks of treatment-DM Sprague-Dawley rats model compared with the normal Sprague-Dawley rats. This result supported the study of Pradhan, *et al.,* in 2015 which stated that elevated levels of CRP and IL-6 can predict the development of type 2 DM and supported a possible role for inflammation in diabetogenesis.[40] In rodent model of glucose metabolism, the *in vivo* infusion of human recombinant IL-6 has been shown to induce gluconeogenesis, subsequent hyperglycemia, and compensatory hyperinsulinemia.[41] Study by Pickup *et al.* (1997) demonstrated that there was an elevated level of IL-6 in individuals with features of the insulin resistance syndrome and clinically T2DM.[42] The effect of IL-6 on hepatic acute phase reactant can also impact atherogenesis. IL-6 treated animals showed significantly increased fibrinogen and decreased albumin when normalized to nontreated controls. Increasing fibrinogen concentration can augment blood clotting whereas decreasing albumin may increase platelet reactivity, both of which could contribute to disease progression in human.[39]

In clinical research, oral Darapladib induced a significant decrease in plasma IL-6.[32,43,44] This is similar with the result of this study that Darapladib can significantly lower the IL-6 expression on the T2DM Sprague-Dawley rats model in 8 weeks of treatment; and in 16 weeks of treatment, there was a decrease trend of the IL-6 expression, although the result is not significant. This is also consistent with *in vivo* studies of Wang *et al.* (2011) which said that oral Darapladib induced a significant decrease of IL-6.[45]

Atherosclerosis contributes to myocardial infarction and stroke, the principal causes of death. Dyslipidemia and inflammation cooperate to promote atherosclerosis progression.[45] Thus, the need to target a new therapeutic target of dyslipidemia is urgently required to decrease cardiovascular risk. This study showed that atherosclerosis development caused by inflammation in T2DM Sprague-Dawley rats could be reduced as the effect inhibiting the new therapeutic target, Lp-PLA2, Darapladib administration. The newest information from this study is that we are able to achieved a more complete and integrated data for IL-1β and IL-6 heart tissue expression pattern in two risk factor model of cardiovascular disease, which is dyslipidemia and T2DM by following chronicity theory of atherosclerotic process using time series experimental design (animal model).

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