Preventive Effects of Alpha-Lipoic Acid on Lipopolysaccharide-Induced Endothelial Dysfunction in Rats

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ABSTRACT
Endothelial dysfunction has been considered as one of the key initial events in the pathogenesis of atherosclerosis and other cardiovascular diseases (CVDs). Several studies imply that chronic inflammation and oxidative stress play a critical role in endothelial dysfunction. The present study was designed to evaluate the preventive effect of alpha-lipoic acid (ALA) on lipopolysaccharide (LPS) induced endothelial dysfunction in rat based on malondialdehyde (MDA) level and vascular cell adhesion molecules-1 (VCAM-1) expression. Thirty Wistar rats were administered ALA for 2 weeks in different doses (30, 60, 120 mg.kg⁻¹ BW) 1 hour before LPS 0.5 mg.kg⁻¹ BW i.p challenge. The LPS was injected on 1st, 4th, and 9th day. MDA plasma level was analyzed with spectrophotometer λ 529 nm, and VCAM-1 expression was determined by immunohistochemistry. Pretreatment with ALA for 14 days could decrease plasma MDA level on LPS-induced endothelial dysfunction in rats. However, only one group of ALA doses, 120 mg.kg⁻¹ BW, showed significant difference with LPS untreated group statistically. It was also found that ALA, in all treatment groups, could attenuate VCAM-1 expression. These findings suggest that ALA had a protective effect against endothelial dysfunction and may potentially prevent vascular inflammatory disease.

Keywords: alpha lipoic acid; lipopolysaccharide; endothelial dysfunction; MDA; VCAM-1

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INTRODUCTION
Cardiovascular diseases (CVDs) are a group of disorders or conditions affecting the heart and vascular diseases of the brain or blood vessels. According to the WHO facts sheet, 31% of all global deaths were mainly caused by CVDs more than any other cause. It was estimated that 17.9 million people died from CVDs in 2016. Of these deaths, 85% are due to heart attack and stroke. More than 75% of CVD deaths occur in low-income and middle-income countries (WHO, 2014). In Indonesia, the proportional mortality caused by CVD reached > 35% in 2016 (WHO, 2018).

The disorders in the cardiovascular system begin with endothelial dysfunction. Endothelial dysfunction is the early predictor of atherosclerosis (Mudau et al., 2012). Cardiovascular risk factors such as hypertension, dyslipidemia, diabetes, inflammation, obesity, and smoking also lead to endothelial dysfunction and contribute to the disease progression (Munzel et al., 2010; Favero et al., 2014). The characteristics of that condition are the increasing of endothelial permeability and production of inflammatory mediators, such as a cytokine, chemokine, and adhesion molecule, especially Vascular Cell Adhesion Molecule-1 (VCAM-1). Endothelial dysfunction is followed by platelet activation, smooth muscle cell proliferation and migration (Favero et al., 2014; Liu et al., 2012; Steyers and Miller, 2014; Gimbrone and Garcia-Cardeña, 2016). All of them can cause the development of atherogenesis, and they are responsible for the death caused by CVDs (Munzel et al., 2010, Wang & Huo, 2010).

Furthermore, oxidative stress is also responsible for inducing endothelial dysfunction. Oxidative stress means a condition in which cells are exposed to reactive oxygen species (ROS) in excessive level. The molecule acts as a messenger molecule that involves vascular inflammation (George & Lyon, 2010; Widlansky & Gutterman, 2011). Malondialdehyde (MDA) is a lipid peroxidation product caused by excessive ROS involved in the pathophysiology of endothelial dysfunction. The previous study showed a positive correlation between MDA and atherogenic index in hyperlipidemia (Yang et al., 2008), preeclampsia (Bharadwaj et al., 2014), and sickle cell nephropathy (Emokpae and Uadia, 2012).

In recent years, antioxidants become one of therapeutic approach to improve endothelial function in preventing CVDs. Alpha-lipoic acid (ALA), also known as lipoic acid is a dithiol compound that acts as an antioxidant. It is synthesized from octanoic acid enzymatically in the mitochondrion. It has a role in energy metabolism as a
Preventive Effects of Alpha-Lipoic Acid

Pharm Sci Res, Vol 6 No 2, 2019 125

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cofactor for mitochondrial α-ketoacid dehydrogenases. It is now marketed widely and has much potential health benefits, especially for preventing diabetic polyneuropathy by improving motor nerve conduction of upper and lower extremities (Vijayakumar et al., 2014). ALA provided a significant improvement in the manifestations of diabetic sensorimotor polyneuropathy (Mcllduff & Rutkove, 2011). Ying et al. (2010) reported that in vitro, ALA was able to inhibit nuclear factor κB (NF-κB) activation, to decrease oxidative stress and expression of adhesion molecules in atherosclerosis model.

Furthermore, ALA improved cardiac function, attenuated VCAM-1 expression and leukocyte migration in myocardial through phosphoinositol-3-Akt (PI3Akt) dependent pathway (Jiang et al., 2013). Unfortunately, there were only a few studies about ALA on lipopolysaccharide (LPS)-induced endothelial dysfunction in rats, especially in a constant challenge. Therefore, this present study was designed to evaluate the effectiveness of ALA on LPS-induced endothelial dysfunction.

METHODS

Materials

Alpha-lipoic acid (ALA) as a sample was obtained from Dexa Medika (Palembang, Indonesia). LPS E. coli 055: B55 and 2-Thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). VCAM-1 antibody, sc-1504 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and kit for immunohistochemistry was purchased from Biocare Medical (USA). Trichloroacetic acid (TCA), NaOH, HCl (Merck, Jakarta, Indonesia), and tetra ethoxy propane (TEP) (TCI, Tokyo, Japan) were used for determining the level of MDA plasma and other reagents, such as diethyl ether, propylene glycol, aqua dest, and formaldehyde were purchased from Bratachem (Surabaya, Indonesia).

Animals

Adult male Wistar rats (150-250 g), 2-3 months of age, healthy were procured from Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia and were housed in suitable cages per group (n=6) at an ambient temperature (25±2°C), relative humidity (50± 5%), with a 12:12 h light-dark cycle (lights on at 07.00 am until 7.00 pm). Animals had free access to standard pellet chow (Japfa Comfeed Indonesia, Surabaya, Indonesia) and drinking water. All animals were adapted for a week in the laboratory. The Animal Ethics Committee, Faculty of Veterinary, Airlangga University, Surabaya, Indonesia has approved this experimental procedure, with document number: 433-KE.

Experimental Protocol

Thirty rats were randomly assigned to 5 groups. Rats in the healthy group received saline i.p. injection, whereas LPS group and all of the treatment groups were induced with LPS 0.5 mg.kg⁻¹ BW in saline i.p, three times induction, once in every three days (1st, 4th, and 9th day). One hour before LPS induction, both the healthy and LPS groups received propylene glycol 50% 1mL/day per oral, whereas the treatment groups received ALA in different doses, 30 mg.kg⁻¹ BW, 60 mg.kg⁻¹ BW, and 120 mg.kg⁻¹ BW per oral. Both ALA or 50% propylene glycol was administered every day for 14 days (1st day to 14th day). On the 15th day, surgery was performed, and blood plasma was collected for determining MDA level. The aortic incision preparation was also obtained and prepared by immunohistochemistry method to measure the VCAM-1 expression.

Determination of plasma MDA level

A volume of 0.5 ml plasma was pipetted and put in a 10 mL test tube, then added with 0.5 ml TCA to precipitate the protein. Briefly, 1 ml Sodium-TBA 1% and HCl 0.1 N were also added until getting 10 mL solution. They were mixed then heated up in a water bath at 90°C for 30 min. Then all the tubes were rapidly cooled and centrifuged at 3000 rpm for 15 min. All procedure for determining plasma MDA level used Thiobarbituric acid-reactive substances (TBARS) methods. The absorbance of the pink solution was measured photometrically using spectrophotometer UV Vis at 529 nm. The concentration was expressed as µmol/L.

Immunohistochemistry and scoring of VCAM-1 expression

Aortic tissue was fixed in 10% formaldehyde for a minimum of 24 hours. Then it was embedded in paraffin wax and was sliced at 4 µm thickness. It was incubated overnight in 4°C with rat antibody VCAM-1. The expression of VCAM-1, appeared dark brown on the surface of endothelial cells, was observed using Light Microscope (Olympus BX53 with camera DP21) and scored using the Allred system. It was calculated by combining the proportion of positive cells and the intensity of the reaction product in most of the examined field. The two scores are then added together to get a final score with eight possible values (Allred, 2005).

Statistical analysis

The results of MDA level data were presented as means ± standard deviation (x ± SD). Between-group comparisons were performed using one-way analysis of variance (ANOVA), followed by Tukey’s procedure for multiple range tests. Scoring of VCAM-1 expression was analyzed with Kruskal-Wallis, continued with Mann-Whitney test. A value of p<0.05 was considered significant.
RESULTS AND DISCUSSION

Endothelial dysfunction breaks the mechanism of vascular homeostasis regulation. It results in vasoconstriction in the blood vessel wall, leading to leukocyte migration and adhesion. It also promotes the activation of platelet, thrombosis, coagulation, oxidative stress, and inflammation then lead to the pathogenesis of cardiovascular diseases (Favero et al., 2014). Endothelial dysfunction has been assumed as one of the critical initial events in the pathogenesis of atherosclerosis and responsible for the death caused by CVDs (Wang & Huo, 2010).

Since LPS can induce inflammation in the vascular wall through direct activation of resident cells such as endothelial cells, this experimental model can be used to cause endothelial dysfunction. The circulating cytokines or other inflammatory mediators that are produced by LPS also develop sickness behaviour syndrome and oxidative stress in rat that can be protected with COX inhibitors (Jayashree & Prakash, 2015). LPS from E. coli has high toxicity. It is often considered as the most potent initiator for activating monokines. The structure of Hexa-acyl diphosphorylated lipid A with acyl chains of length C12–C14 is widely believed to the structure optimally configured to stimulate maximal activation of toll-like receptor-4 (TLR-4). This complex will then lead to monocyte activation (Erridge et al., 2002).

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\text{In vivo, LPS E. coli (0.05 mg.mL}^{-1}\text{ LPS in PBS or 0.5 mg.kg}^{-1}\text{ BW i.p)}\text{ could induce endothelial dysfunction in 24 hours through increasing protein expression in endothelial cells, such as LOX-1, NFκB, and p38- mitogen-activated protein kinase (MAPK) (Zhao et al., 2014). The LPS-mediated inflammatory environment also affects iNOS, TNFα and IL-6 mRNA levels in a concentration-dependent manner (Baek et al., 2018). Since it was known that LD50 of E. coli LPS i.p in the rat is 5 mg.kg}^{-1}\text{ BW, and minimum dose 1 mg.kg}^{-1}\text{ BW still caused some deaths in 24 hours (Ashour et al., 2011), thus in this present study LPS dose 0.5 mg.kg}^{-1}\text{ BW was used. Three times inductions, once in every three days, are being considered to obtain the persistent effect of endothelial dysfunction.}
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Effect of ALA on plasma MDA level

A growing body of evidence suggests that increased production of ROS can induce endothelial dysfunction (Munzel et al., 2010). LPS-induced rabbits showed increased production of protein oxidation (carbonyl) and lipid peroxidation (MDA & 4-hydroxy alkene) both in tissue or plasma (Gonzalo et al., 2010). The mechanism explained that a complex of LPS/TLR-4 would trigger intracellular ROS production mediated by NADPH oxidase and through activation NFκB and MAPK pathway will induce the increasing of pro-inflammatory mediators. The increasing of MDA level will reduce endothelial function (Lee et al., 2012).

The result of this study showed that three times inductions of LPS 0.5 mg.kg\(^{-1}\) BW i.p, once in every three days induced oxidative stress as shown by significant increase of MDA level (p<0.001), almost two folds compared to the healthy group. Treatment with ALA at a dose 30 mg.kg\(^{-1}\) BW, 60 mg.kg\(^{-1}\) BW, and 120 mg.kg\(^{-1}\) BW for 14 days administered 1 hour before LPS challenge decreased plasma MDA level that was dose-dependent.

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\text{Figure 1 - Effects of ALA on plasma MDA mean concentration in rats}
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LPS 0.5mg.kg\(^{-1}\) BW i.p was induced three times, once every three days (in 1st, 4th, and 7th day). ALA at dose 30; 60; 120 mg.kg\(^{-1}\) BW was administered by oral gavage, every day for 14 days, 1 hour before LPS challenge. Plasma MDA level was measured by the TBARS method using spectrophotometer UV-Vis λ,529 nm. MDA concentration was shown as mean ± SD (n=5). *p<0.001 vs healthy group; #p<0.001 vs LPS group (Tukey’s multiple comparison tests).
There was a significant difference observed at p<0.001 (Figure 1). Tukey’s tests were then performed, showing only ALA 120 mg.kg\(^{-1}\) BB was statistically significant compared to LPS untreated group (p<0.001), but it was not significant compared to the healthy group (p=0.677).

The decreasing plasma MDA level, especially after 14 days of treatment with ALA would decrease oxidative stress. As a biological antioxidant, ALA has two mechanisms, direct and indirect activities. As a direct antioxidant, ALA and its reduction form, dihydrolipoic acid (DHLA) scavenge hydroxyl radicals, singlet oxygen, and hypochlorous acid. They also regenerate endogenous antioxidants such as vitamins C and E, and GSH by reducing the oxidized forms. Whereas as an indirect antioxidant, both ALA & DHLA have metal-chelating properties due to dithiolane ring’s activities. In vitro, lipoic acid can chelate of divalent metal ions and form stable complexes with Zn\(^{2+}\), Cu\(^{2+}\), Mn\(^{2+}\), and Fe\(^{2+}\) (Goraca et al., 2011; Rochette et al., 2015). The other study showed that preincubation of the HUVECs with ALA increased cell viability, all oxLDL effect, and decreased MDA content (Liu, 2011).

In vivo, in LPS-induced model, ALA decreased tissue MDA level & H\(_2\)O\(_2\) in lymph, brain, heart (Goraca et al., 2011; Goraca et al., 2013). In the sepsis model, ALA balanced redox by decreasing testis MDA, NO, 8-hydroxydeoxyguanosine (8-OHdG) level (Ashour et al., 2011). It could also improve endothelial function due to reducing MDA level both in plasma and aortic tissue in a diabetic model (Ying et al., 2010), ischemic myocardial (Wang et al., 2011) and hypercholesterolemia rat (Sari et al., 2016).

**Effect of ALA on VCAM-1 expression**

Adhesion molecules were also essential markers of endothelial dysfunction. Several mediators, including microbial stimulation of TLRs, production of cytokines, excessive levels of ROS, or turbulent blood flow at vessel bifurcations induce the expression of VCAM-1 (Milis et al., 2011). The up-regulation of VCAM-1 is a critical step in leukocyte or monocyte migration to intima during inflammation (Wu et al., 2012). Lipopolysaccharide-induced VCAM-1 expression (Jiang et al., 2013) and leukocyte adhesion in lung tissue (Wu et al., 2012). In vitro, LPS also promoted VCAM-1 expression and other adhesion molecules in human umbilical cord vein cell (HUVEC) (Liu et al., 2012; Wu et al., 2012, Baek et al., 2018), mesangial cell (Lee et al., 2012); vascular smooth muscle cell (Meng et al., 2013); mouse glial and neuronal cultures (Leow-Dyke et al., 2012). The mechanism involves TLR-4 stimulation, then trigger...
ROS intracellular production and MAPK & NFκB pathway activation (Liu et al., 2012; Lee et al., 2012; Wu et al., 2012; Burger & Touyz, 2012). Finally, they induce LOX-1 expression via TLR4/MyD88/ROS, then activated the p38MAPK-NF-κB pathway to promotes ox-LDL endocytosis and acts as an adhesion molecular supporting endothelial-monocyte interaction (Zhao et al., 2014).

By understanding the molecular mechanism of vascular inflammation in atherosclerosis are now yielding novel target for pharmacotherapy (Rahmathulla & Devi, 2015). Since ROS could induce VCAM-1 production and expression, antioxidants were believed as a potential agent in endothelial dysfunction by blocking its signal transduction, and VCAM-1-dependent inflammation. The signalling of VCAM-1 became a target for intervention by antioxidants or by pharmacological agents during inflammatory diseases (Mills et al., 2011), especially in cardiovascular disease.

The VCAM-1 expression on the surface of endothelial cells was determined by staining aortic tissue with rat antibody VCAM-1 (Figure 2). In a healthy group, negative staining of aortic tissue was observed (Figure 2A). Positive staining was demonstrated by dark brown (noted by black arrow) on the surface of endothelial cells from all groups threat with LPS 0.5 mg.kg⁻¹ BW. The most robust expression was shown in the untreated group (Figure 2B). The protein expression on the endothelial cell surface was attenuated after 14 days ALA administration in LPS challenge rats, depending on the dose, as illustrated in Figure 2C (ALA 30 mg.kg⁻¹ BW), Figure 2D (ALA 60 mg.kg⁻¹ BW), and Figure 2E (ALA 120 mg.kg⁻¹ BW). The scoring of VCAM-1 expression, which determined by the Allred system, interpreted the weak staining in all ALA treatment groups (data was not shown). Then, analysis with Kruskal-Wallis showed that ALA administration in the treatment group (30, 60, and 120 mg.kg⁻¹ BW) significantly attenuated VCAM-1 expression (p<0.001).

This present study showed that ALA administration, as one of the potential antioxidant could attenuate VCAM-1 expression. In line with this result, in vitro assays using HUVEC (Tomo et al., 2015) and homocysteine-induced injuries to human aortic endothelial cells (HAECs) (Hu et al., 2016). Whereas in vivo, ALA treatment markedly and dose-dependently attenuated the expression of VCAM-1 in ovariectomized Ldlr⁻/- mice (Shen et al., 2018). Lipoic acid decreased oxidative stress, inhibited NF-κB activation, improved vascular reactivity, attenuated expression of adhesion molecules, especially mRNA expression of VCAM-1 in thoracic aorta of Watanabe heritable hyperlipidemic (WHHL) and also reduce atherosclerotic plaques in the abdominal aorta in rabbits (Ying et al., 2010) and atherosclerotic mice (Lee et al., 2012).

The arrangement of ALA in attenuating VCAM-1 expression involves the activation of the PI3K/Akt pathway that can negatively regulate the NFκB activation pathway. Previous studies showed that ALA ameliorated inflammation and apoptosis in aortic tissues of aged type II diabetes rat (Bitar et al., 2010) and attenuated LPS-induced cardiac dysfunction (Jiang et al., 2013) through a PI3K/Akt-dependent mechanism. The PI3K/Akt pathway can negatively regulate the NFκB activation pathway. Lipoic acid also inhibited IκBα degradation and NFκB activation through inhibition of IKK2 rather than its antioxidant function (Ying et al., 2011). In contrast, lipoic acid as an antioxidant may also oxidize sulfhydryl groups or form mixed disulfides on proteins, thus influencing the changes in the thiol redox status of NFκB than its antioxidant function (Ying et al., 2011). In contrast, lipoic acid as an antioxidant may also oxidize sulfhydryl groups or form mixed disulfides on proteins, thus influencing the changes in the thiol redox status of NFκB as a redox-sensitive transcription factor (Goraca et al., 2011). The suppression of NFκB will lead to a decrease in the production of pro-inflammatory genes, especially VCAM-1. Thus, the inflammation responses were also limited as the decreasing of the VCAM-1 output.

CONCLUSION

In conclusion, ALA may have a protective effect against LPS-induced endothelial dysfunction in rats and have a potential health benefit in preventing inflammatory vascular disease. However, further studies in detail are required to explore other mechanisms of ALA in preventing endothelial dysfunction.

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Preventive Effects of Alpha-Lipoic Acid

Pharm Sci Res, Vol 6 No 2, 2019 129


