Vascular remodeling and association with inflammation in the heart of obesity model

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ABSTRACT

Introduction: Obesity alters several metabolic activities, subsequently leading to the development of cardiovascular diseases. The insulin resistance-induced obesity stimulates vasodilatation and vasoconstriction imbalance, which ends up in cardiac vascular remodeling. Therefore, we aimed to investigate the effect of obesity in cardiac diseases with a focus on inflammatory mediators associated with endothelial dysfunction.

Materials and Methods: Rats (3 months old, weighing 200 g) were divided into control (n=6) and the obese groups, which included rats fed on a high-fat diet (HFD, n=6 in each sub-group) for 1 month (OB1), 2 months (OB2), and 4 months (OB4). Then, the rats were sacrificed, and their hearts were harvested for histological quantification as well as the quantification of the mRNA expression of inflammatory mediators, eNOS, and ppET-1 by reverse transcriptase-polymerase chain reaction (RT-PCR). Sirius Red staining was performed to assess vascular remodeling, while immunohistochemistry of CD68 was performed to assess the localization of macrophage.

Results: HFD-induced obesity was significantly manifested in the obese groups relative to that in the control group. It was followed by an increase in the mRNA expression of inflammatory mediators in the obese groups when compared to that in the control group. Long-term obesity promoted vascular remodeling, which was noted in the OB4 group, along with downregulation of the eNOS mRNA expression and the upregulation of the ppET-1 mRNA expression.

Conclusion: Obesity associated with inflammation and vascular remodeling in the heart.

KEYWORDS:
Obesity, inflammatory mediators, vascular remodeling, eNOS, ppET-1

INTRODUCTION

Obesity is a condition marked by the presence of excess fat tissues in several areas of the body that results in a high body mass index (BMI). It has been known that people with BMI >30 are categorized as obese and that they may be at a greater risk to several health problems.[1,2] The prevalence of obesity has increased over several past decades in both developed and developing countries. In 2013, more than 50% of the population in Oceania, North Africa, and the Middle East countries were recorded as obese.[3] According to The Indonesian National Basic Health Research, the prevalence of people with BMI score >27 in Indonesia has increased since 2007, namely by 10.5%, 14.5%, and 21.8% in the years 2007, 2013, and 2018 respectively.[4,5]

Obesity is associated with heart failure, atrial fibrillation, sudden cardiac death, and myocardial steatosis.[6,7,8,9] High BMI correlates with a high level of fat mass and free fatty acid (FFA), which has a severe effect on the cardiovascular system.[10] An accumulation of adipose tissue increases the release of adipokines[11] and pro-inflammatory cytokines, such as IL-6 and TNF-α,[12] which results in the infiltration of immune cells.[13] Hypertrophy and hyperplasia of the adipose tissues lead to lipotoxicity and alter lipid metabolism. An increase in the lipid metabolism promotes macrophage infiltration and activation mediated by nuclear factor kappa-light-chain-enhancer of the activated B cells (NF-κB) pathway. An increase in the level of FFA, cardiomyocyte fatty acid uptake exceeds the mitochondrial oxidative capacity, ultimately forming inclusions of lipid within the cardiomyocytes, also known as cardiac steatosis. This condition leads to lipotoxicity of the hearts.[14]

Inflammation contributes to the development of early endothelial dysfunction that leads to the development of atherosclerotic plaque. The acute phase protein and inflammatory mediators in circulation aggravates myocardial fibrosis and endothelial dysfunction. Imbalance vasodilator and vasoconstrictor agent are regulated by phosphatidylinositol 3-kinase-dependent (PI3K) and mitogen-activated protein kinase, respectively, in vascular endothelial tissues. Measuring the diameter and blood flow in coronary arteries is regarded as the gold standard to assess endothelial function.[15,16] This study was performed to explore the effect of obesity in the cardiovascular system with a focus on inflammatory mediators associated with endothelial dysfunction.

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MATERIALS AND METHODS

Animal study

This study was conducted according to the ethical approval of the Ethical Committee of Medical Research and Health of Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (ethical expediency number KE/FK/0385/EC/2019). The rats were maintained at 25°C–30°C with 50%–60% humidity, under a dark-light cycle of 12:12 h.

Sprague–Dawley rats (age: 3 months, weight: 180–200 g) were divided into 4 groups, namely control group that received standardized food (Control, n = 6) and obese groups that were fed with a high-fat diet (HFD). The obese groups consisted of rats with obesity for 1-month (n = 6, OB1), obesity for 2 months (n = 6, OB2), and obesity for 4 months (n = 6, OB4) groups. Lee Index (($\sqrt[3]{}$weight(grams))/naso-anal length (mm)) was used to determine the level of obesity. At the due date, the rats were sacrificed, and their hearts were harvested. The area under the coronary sulcus was immersed in an RNA preservation solution (Favorgen, FARSS100) for RNA extraction, and the apex was immersed in 4% paraformaldehyde.

RNA Extraction and cDNA synthesis

The heart of rat was extracted according to the procedural technique described by the manufacturer of the Genezol RNA Solution (GENEzol™, Cat. No. GZR100). Then, 3000 ng of total RNA was used to synthesis the cDNA. The synthesis of cDNA was performed using the cDNA Synthesis Kit (SMOBio, RP1400) with the PCR condition of 25°C for 10 min, 42°C for 50 min, and 85°C for 5 min.

Reverse transcriptase PCR (RT-PCR)

Assessment of the mRNA expression of inflammatory mediators was done using CD68 (forward 5′-TGTGTCCTTCCCACAAGCAG-3′ and reverse 5′-AAGAGAAGCATGGCCCGAAG-3′), NFκB (forward 5′- CACTCTCTTTTTGGAGGT-3′ and reverse 5′-TGGATATAAGGCTTTACG-3′), and MCP-1 (forward 5′-GCTGTAGTATTTGTCACCAAGCTC-3′ and reverse 5′-ACAGAAGTGCTTGAGGTGGTT-3′). The mRNA expression of eNOS was assessed using forward 5′-CCGGCGCTACGAAGAATG-3′ and reverse 5′-AGTGCCACGGATGGAAATT-3′) and the ppET-1 performed using forward 5′- GTCGTCCCGTATGGACTAGG-3′ and reverse 5′-ACTGGCATCTGTTCCCTTGG-3′). PCR conditions were denaturation at 94°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, followed by the final extension phase end step at 72°C for 10 min. The RT-PCR was performed by mixing cDNA, Taq master mix (Promega, GoTaq Green, M7122), and primers. The PCR products were analyzed on 2% agarose gel along with a 100-bp DNA ladder (Bioron, Germany, Cat. No. 306009). The internal control used HRPT-1 (forward 5′- AGACGTTCTAGTCCTGTGGC-3′ and reverse 5′- ATCAAAAGGGACGCAGCAAC-3′) and β-actin (forward 5′-GCAGATGTGGATCAGCAAGC-3′ and reverse 5′- GGTGTAAAACGCAGCTCAGTAA-3′), was used to normalize the expression.

Immunohistochemistry staining of CD68

The samples were cut into 4-µm-thick paraffin sections and placed in coated-object glass, followed by deparaffinizing with xylene and rehydration with grading alcohol. Next, the antigen was retrieved using heat-induced antigen retrieval methods, followed by blocking peroxidase with 3% H202 in the PBS solution. Then, the slides were incubated with the blocking serum for 20 min and incubated with mouse 1st polyclonal antibody anti-CD68 (Abcam®, ab955; 1:300) overnight. On the following days, the slides were incubated with antibodies (Biocare Medical®, STUHRP700L10), and diaminobenzidine tetrahydrochloride (DAB) (Biocare Medical®, STUHRP700L10). The results were captured under a light microscope (Olympus CX22®) through the Optilab software with 400x magnification.

Statistical analysis

Data were analyzed using the SPSS 22 software, and the normality test was performed by using Shapiro–Wilk. Normally distributed data were analyzed using One-way ANOVA and independent t-test. The significant value was determined if the *p*-value was less than 0.05.

RESULTS

HFD feeding stimulated obesity in conjunction with the severity of cardiac inflammation

According to the Lee Index, at the end of study, we showed that HFD feeding for 1, 2, and 4 months significantly caused obesity in Sprague-Dawley rats ($p = 0.000$). Starting at week 8, the body weight increased gradually, which was noted in OB2 ($p = 0.009$) and OB4 ($p = 0.006$) when compared to that in the control group. At week 12 and week 16, a remarkable escalation of the body weight in OB4 was noted to that in the control group ($p = 0.000$).

With an increase in the body weight, the inflammatory cytokines were upregulated. Long-term obesity provoked cardiac inflammation, which was obvious in the OB4 groups. The mRNA expression of NFκB, MCP1, and CD68 in the OB4 group was markedly elevated when compared to that in the control group ($p < 0.05$). However, the mRNA expression of NFκB and MCP1 in the OB1 group was not statistically different when compared to that in the control group (Fig. 1).

Obesity enhanced cardiac vascular remodeling via an imbalance of vasodilator and vasoconstrictor agent

Finally, to evaluate the functional alteration induced by obesity, our results suggested a significant difference between the control and OB groups ($p < 0.01$). This alteration was accompanied by the downregulation of eNOS mRNA expression and the upregulation of ppET-1 mRNA expression in obese groups, particularly in the OB4 group ($p < 0.05$) (Fig. 2).

DISCUSSION

Our results demonstrated that an HFD stimulates vascular remodeling mediated by the upregulation of inflammatory cytokines. It has been understood that obesity-induced low-grade inflammation as a result of the enlargement of adipocytes that leads to hypoxia and reactive oxygen species (ROS) development.[17,18] The circulating inflammatory mediators contribute to the development of cardiac inflammation in a time-dependent manner (Fig. 1). Abdominal white adipocyte tissue inflammation has also

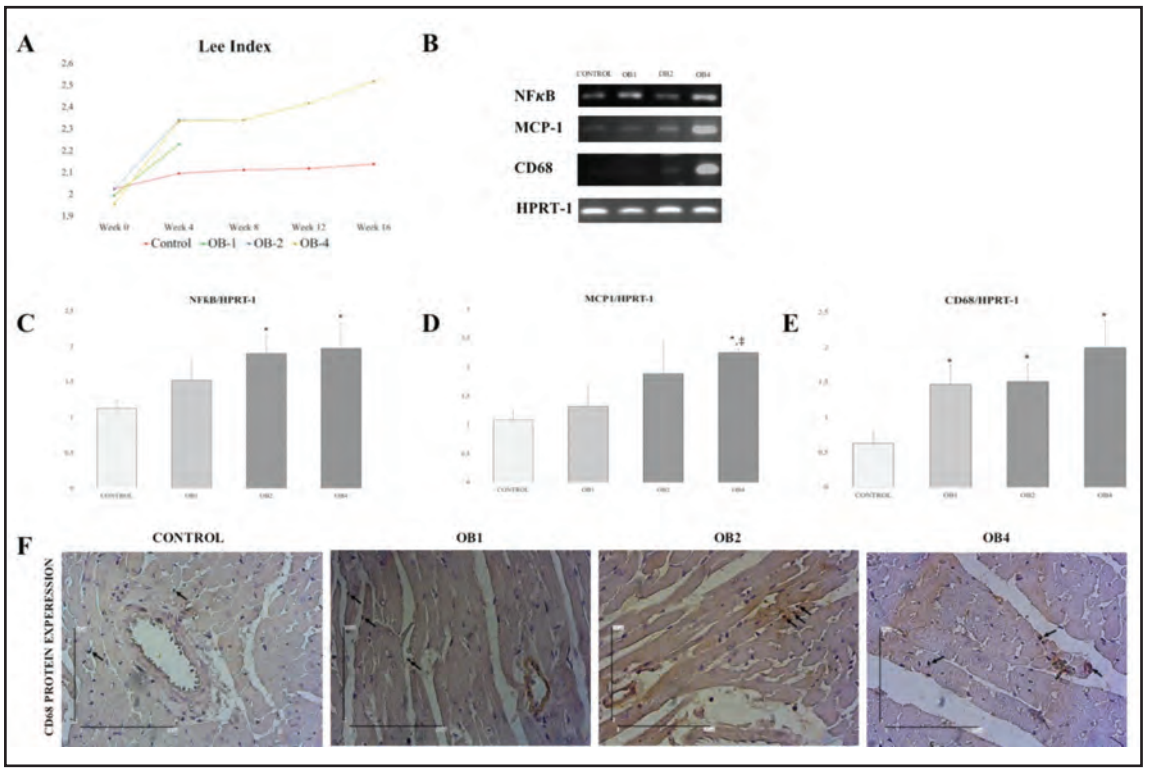

Fig. 1: Obesity associated with an increase in the level of inflammatory mediators. A. Bar chart of Lee Index. B. Representative figures of inflammatory mediators. C–E. Quantification of NFκB, MCP-1, and CD68 mRNA expression. F. CD68 protein expression.

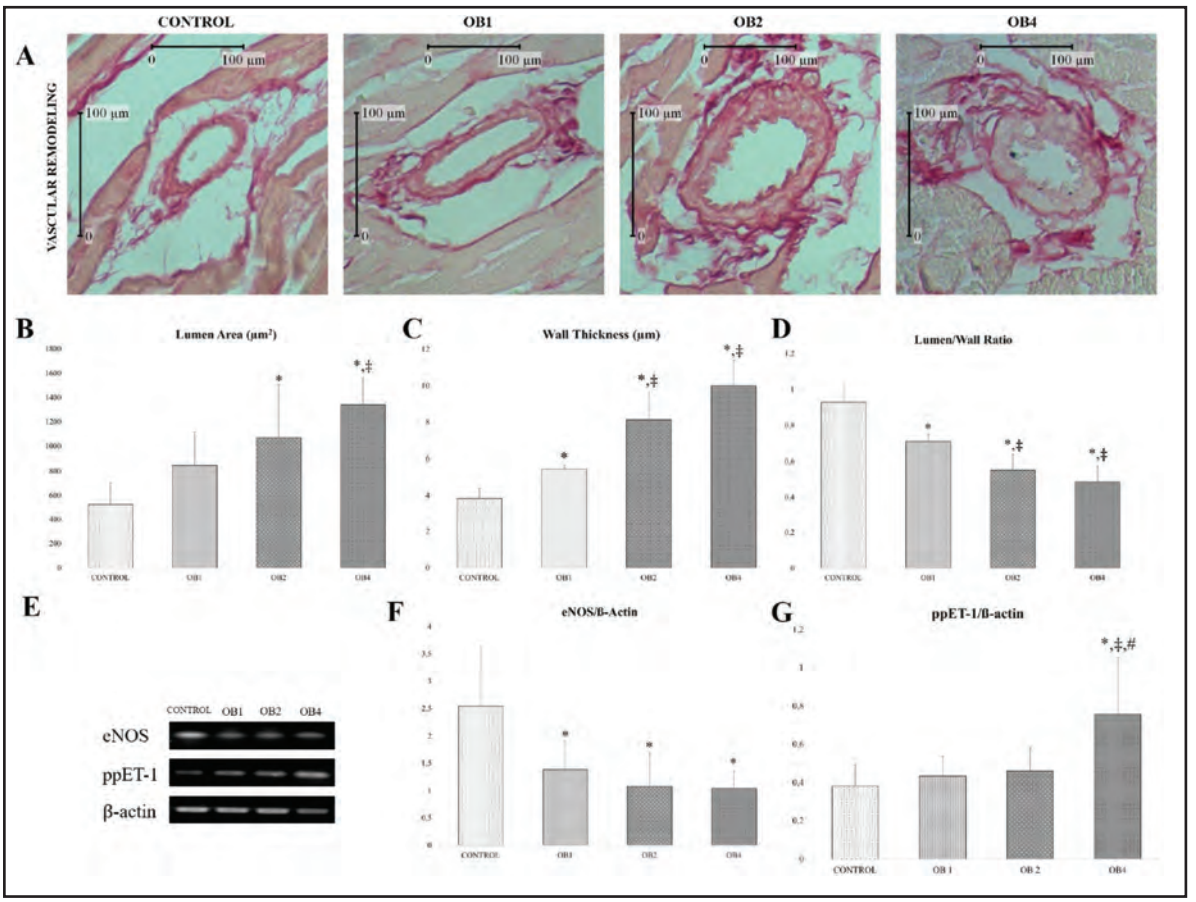

Fig. 2: Obesity-induced vascular remodeling. A. Representative figures of vascular remodeling by Sirius Red staining. B. Quantification of the lumen area. C. Quantification of the wall thickness. D. Quantification of the lumen/wall ratio. E. Representative figures of the eNOS and ppET mRNA expression. F. Quantification of the eNOS mRNA expression. G. Quantification of the eNOS mRNA expression.

been correlated with the development of cardiovascular diseases, such as atherosclerosis and diabetes mellitus type 2. The nuclear factor κB (NFκB) luciferase reporter assay activity in an HFD showed markedly higher activity when compared to that in the abdominal region of low-fat-diet-fed male mice.[19] In obese db/db mice, the blockade of NFκB alleviated the oxidative stress and improved the cardiac function.[18] The FFA secreted by adipocyte tissues are recognized by Toll-like receptor 2/4 (TLR2/4), which is highly expressed in the cardiac tissues of HFD-induced mice model. Downstream of TLR, the activation of MyD88, an adaptor protein, plays a crucial role in inducing pro-inflammatory cytokine through the phosphorylation of IκB, which eventually leads to nuclear translocation of NFκB and the production of pro-inflammatory genes expression, including MCP-1.[20–22] Hypoxia and oxidative stress promote cardiac inflammation through HIF-1α. HFD feeding enhances the upregulation of mRNA and protein HIF-1α in adipocyte tissues in a time-dependent manner.[23] The crosstalk between NFκB and HIF-1α has been well-documented. The proximal promotor of HIF-1α contained a NFκB binding-site at -197/-188 bp under hypoxia. This condition promotes an increase in the NFκB activity and enhanced inflammatory response.[24,25]

Saturated fatty acid *per se* induces the NLRP3 inflammasome activation, which leads to IL-1β release in macrophage cell culture in a dose-dependent manner.[26] Macrophage and adipocyte cells expressed an abundance of CCR2 under metabolic disorders. This increment is in accordance with the elevation of CCL2 or MCP1 in the adipose tissues of obese rodents.[27–29] The circulating MCP-1 extricated by adipose tissues were doubled in obese diabetic (db/db) mice model when compared to that in lean mice as a control. An elevation of circulating MCP-1 level was positively correlated with an elevation of BMI, waist circumference, IL-6, and HOMA and negatively correlated with the HDL-cholesterol level.[30] Our result suggests that an elevation of MCP-1 was demonstrated in HFD feeding for 4 months (Fig. 1). Higher MCP-1 level has been strongly correlated to the development of atherosclerosis.[30,31] It promotes the recruitments of monocytes into the subendothelial layers. After entering the subendothelial layers, monocytes differentiate into macrophages that produce foam cells of the fatty streak. The deletion of CCR2 has been correlated with the attenuation of macrophage accumulation in the atherosclerotic lesion in apoE null mice with HFD feeding.[32,33]

CD68 is a glycosylated type-1 transmembrane glycoprotein that is mostly expressed by macrophages, other mononuclear phagocytes, and endothelial cells mainly located in the endosomal compartment. Upon responding to the inflammatory stimuli, CD68 was upregulated, and it is demonstrated an ability to bind modified LDL, phosphatidylserine, and apoptotic cells.[34] Long-term inflammation accelerates an increase in M1 macrophage polarization, which in turn markedly increases the CD68 expression. Injection of inflammatory proteins, TNFα, demonstrated a significantly higher intimal plaque formation in accordance with an increase in the CD68 protein expression.[35] In addition, an excess of oxidative stress, DNA damage, high glucose, and ceramides present in a chronic state of obesity, can develop cellular senescence, which produces senescence-associated secretory phenotype (SASP). This SASP can stimulate further inflammation by secreting more IL- 6, TNF-α, MCP1, and other cytokines. With other cellular senescence effects such as extracellular matrix dysfunction and pathological angiogenesis, the accumulation of cells undergoing cellular senescence may lead to aging or age-related disorders, including heart failure and atherosclerotic diseases.[36]

Obesity is commonly accompanied by insulin resistance, the elevation of leptin, and an alteration of the renin-angiotensin system that may cause sodium retention resulting in increased fluid volume which increases markedly by dilatation of the artery. Although the vein is known as the capacitance vessel, increased fluid volume causes dilation of the arterial lumen under the condition of increased body fluid volume. The disturbing vasodilation effect of chronic inflammation may occur in a longer obesity period, which ideally occurs in 6 months, although the longest obesity period in this study was only 4 months.[37–39] However, our results demonstrated thickening of the vascular wall in the obese groups (Fig. 2). In response to the higher lumen area, the wall thickens to compensate for luminal enlargement in order to normalize the stress on the wall.[40] Other than compensating for the wall stress, an increased level of insulin may increase the arterial wall thickness through the direct trophic effect of smooth muscle cells, the generation of ROS, protein kinase C, and activation of NFκB, which stimulates the growth and proliferation of vascular smooth muscle cells.[41] Metabolic changes due to obesity enhances the downregulation of the PI3K/Akt pathway, resulting in a decrease eNOS mRNA expression in 2 months obese-prone Sprague–Dawley rats fed on a HFD.[42]

Deposition of foam cells may increase the vascular wall thickness, which then stimulates the proliferation of vascular smooth muscle cells. The proliferation of vascular smooth muscle is often associated with an imbalance of vasoconstrictor and vasodilator agents represented by the upregulation of ppET-1 mRNA expression and the downregulation of eNOS mRNA expression in long-term obesity. Long-term obesity shown by the OB4 group involved enhanced upregulation of the ppET-1 mRNA expression caused by increased oxidative stress and chronic inflammation.[43,44]

CONCLUSION

Obesity associated with inflammation and vascular remodeling in the heart.

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