Inhibition of Senescence Through Decreasing P16^{ink4a} Expression by Sirt-1 in ADMA Exposed EPC

Titin Andri Wihastuti[1(✉)], Wiwit Nurwidyaningtyas[2], and Kumboyono Kumboyono[3]

[1] Basic Nursing Department, Faculty of Health Sciences, Universitas Brawijaya, Malang, Indonesia
wihastuti.fk@ub.ac.id

[2] Department Molecular and Cellular Biology, Sekolah Tinggi Ilmu Kesehatan Kendedes, Malang, Indonesia

[3] School of Nursing, Faculty of Health Sciences, Universitas Brawijaya, Malang, Indonesia

Abstract. Senescence is associated with various degenerative diseases, such as cardiovascular disease (CVD). The community must bear the economic burden due to CVD, not only from the prohibitive medical costs but also from the decline in people's work productivity due to suffering from CVD. Various efforts have been made to prevent premature senescence. Sirtuin-1 (SIRT-1) is essential in maintaining vascular homeostasis through the modulation of senescence-associated signaling pathways. Vascular homeostasis is highly dependent on the quality of endothelial cells as the primary vascular component. Good vascular regeneration is primarily determined by the Endothelial Progenitor Cell (EPC). Exposure to CVD risk factors is thought to trigger premature senescence of EPC. The molecular mechanism of premature EPC senescence associated with CVD is still unclear. This study aimed to test whether the specific activator of SIRT-1 could inhibit the senescence of EPCs exposed to Asymmetric Dimethylarginine (ADMA) by decreasing P16^{INK4a}, which is one of the markers of cell senescence. True-experimental research method in vitro using EPC culture obtained from PBMNC. This study has three groups: the EPC group, the EPC group with exposure to ADMA, and the EPC group receiving SIRT-1 before exposure to ADMA. The results showed that the intensity of P16^{INK4a} expression increased dramatically in EPCs exposed to ADMA compared to controls. In addition, the study results also showed a decrease in the expression of P16^{INK4a} in EPCs given SIRT-1 before exposure to ADMA compared to EPCs exposed to ADMA without SIRT-1 administration. The decrease indicates the protective effect of SIRT-1 against EPC senescence due to ADMA exposure.

Keywords: atherosclerosis · senescence · EPC · SIRT-1 · ADMA · P16^{INK4a}

1 Introduction

Senescence is associated with various degenerative diseases, such as cardiovascular disease (CVD). The community must bear the economic burden due to CVD, not only

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from the prohibitive medical costs but also from the decline in people's work productivity due to suffering from CVD. Various efforts have been made to prevent premature senescence. Sirtuin-1 (SIRT-1) is essential in maintaining vascular homeostasis through the modulation of senescence-associated signaling pathways. Vascular homeostasis is highly dependent on the quality of endothelial cells as the primary vascular component. Good vascular regeneration is primarily determined by the Endothelial Progenitor Cell (EPC). Exposure to CVD risk factors is thought to trigger premature senescence of EPC. The molecular mechanism of premature EPC senescence associated with CVD is still unclear.

Cell senescence is characterized by irreversible cessation of the cell cycle due to stress induction, which is widely associated with organ dysfunction and diseases associated with senescence [1]. Under normal physiological conditions, old cells can be eliminated by the immune system. However, with increasing age or chronic disease, senescent cells accumulate in tissues, interfere with functional maintenance, increase pathological conditions, and cause maladaptive responses [2].

Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), is associated with impaired endothelial function in humans [3]. In addition, clinical evidence suggests that plasma ADMA levels increase with age or in people with hypercholesterolemia, atherosclerosis, hypertension, chronic heart failure, diabetes mellitus, and chronic renal failure, all of which are significant contributors to endothelial dysfunction and vascular disorders [4, 5]. Our previous study proved that ADMA exposure could induce senescence effector activity in vitro, characterized by an increase in the number of progenitor endothelial cells expressing P16^{INK4a} [6].

The results of previous studies in mammalian cells showed that SIRT-1 decreased the expression of P16^{INK4a} mRNA, a molecular marker of cell senescence and DNA damage [7]. SIRT-1 is said to be involved in inflammatory processes, premature senescence, telomere irritation, secretion of senescence-associated substances, and responses to DNA damage [8]. Based on these exposures, this study aimed to test whether the specific activator of SIRT-1 could inhibit the senescence of EPCs exposed to ADMA by decreasing the expression of P16^{INK4a}. It is hoped that the results of this study can strengthen the evidence for the role of SIRT-1 in anti-premature senescence, especially in EPC, to reduce the risk of CVD. SIRT-1 is naturally contained in many vegetables and fruits. Finally, this research can also be the basis for an exploratory study of vegetable and fruit ingredients in Indonesia that have the potential as natural SIRT-1 activators as candidates for an herbal drug to prevent premature vascular senescence.

2 Method

2.1 Study Design

This study uses a true-experimental laboratory design in vitro, carried out at the Central Laboratory of Biological Sciences, Universitas Brawijaya. This research procedure has obtained ethical feasibility from the Bioscience ethics committee of Universitas Brawijaya No 1206-KEP-UB.

2.2 Sample Collection and Preparation

The procedure for taking peripheral blood and isolating peripheral blood mononuclear cells (PBMNC) in this study is the same as the previous procedure [6, 9].

2.3 EPC Cell Culture, SIRT-1 Administration, and Induction of Cell Senescence

PBMNCs were cultured in Endothelial Growth Medium (EGM) plus 10% FBS at 37 °C with a mixture of 95%: 5% (v/v) moistened with air and CO2. EPC cultures were given SIRT-1 (Select, Shanghai, China) for 24 h to prevent cell senescence. SIRT-1 was previously dissolved in DMSO and applied to reach a final concentration of 10M. To induce senescence of EPC cells, PBMNCs were exposed to ADMA (Sigma, St. Louis) for 24 h. Previously ADMA was dissolved In Phosphate-Buffered Saline (PBS) and used at a concentration of 300 M.

2.4 Identification of $P16^{INK4a}$

After exposure to SIRT-1 and ADMA, cells were washed twice with PBS, then fixed and stained with $P16^{INK4a}$ Staining Kit (Beyotime Institute of Biotechnology, Shanghai, China), and then analyzed with a confocal laser scanning microscope.

2.5 Data Analysis

The researcher tested the hypothesis using the Kruskal–Wallis and followed by a Bonferroni post-hoc test to identify differences between groups (p-Value < 0.05 was considered significant). All data analysis was carried out using STATA software version 14.

3 Results and Discussion

Efforts to delay the incidence of diseases due to premature aging can increase life expectancy. Improving the quality of human life will reduce the burden on the health care system so that people's productivity to support the economy will increase. SIRT-1 increases metabolic activity and protects against physiological disorders due to aging [10, 11], thereby inhibiting EPC aging and reducing the risk of vascular dysregulation, atherosclerosis, and CVD [12–14]. We administered SIRT-1 before 300M ADMA exposure to prove the protective effect of SIRT-1 on ADMA-exposed EPCs. The intensity of $P16^{INK4a}$ expression was significantly decreased in EPCs compared to EPCs not treated with SIRT-1.

3.1 ADMA Exposure Leads to Increased Intracellular P16ink4a Expression

PBMNCs were seeded in culture media for seven days, then labeled into three groups, (i) untreated cells, (ii) cells treated with ADMA for 24 h, and (iii) group given pretreatment activator SIRT1 (SIRT1720) for 3 h before exposure to ADMA for 24 h. As shown in Fig. 1, the expression intensity of $p16^{INK4a}$ increased dramatically in cells exposed to

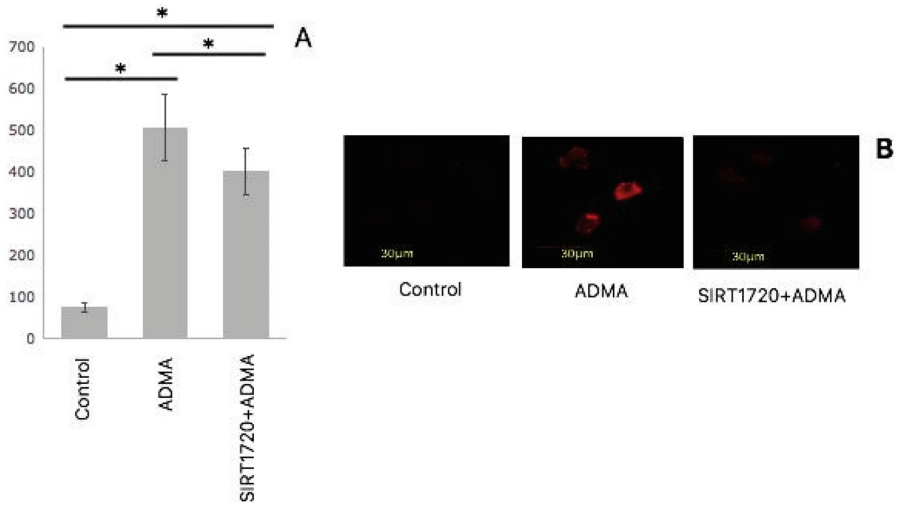

Fig. 1. Representative results of quantification of intracellular p16^{INK4a} expression intensity in EPC. (A) Differences in the average intensity of p16^{INK4a} in each EPC group were observed; (B) Rhodamine staining representing the intensity of p16^{INK4a} in the untreated cell group (control), the cell group exposed to ADMA and the cell group given SIRT1 activator followed by ADMA exposure. Quantification of p16^{INK4a} intensity was validated through a confocal laser scanning microscope. *p-Value < 0.001.

ADMA compared to controls (Fig. 1A), and the condition was improved by pretreatment of the SIRT-1 activator.

ADMA exposure leads to increased intracellular P16^{INK4a} expression. Figure 1(B) shows the proportion of the intensity of P16^{INK4a} expression, which dramatically increased in progenitor endothelial cells exposed to ADMA compared to control/Fig. 1(A).

3.2 Protective Effect of SRT-1 Against ADMA Exposure on EPC

To prove the protective effect of SIRT-1 on ADMA-exposed EPC, we administered SIRT-1 before exposure to 300M ADMA. The expression intensity of p16^{INK4a} decreased significantly in EPC compared to EPC that was not given SIRT-1. The study's results prove that exposure to a 300 M dose of ADMA induces aging effector acceleration in EPC. Several longitudinal studies have revealed that ADMA as an NO inhibitor causes a decrease in telomerase activity which physiologically plays a role in maintaining genomic stability by protecting against chromosome degradation [15–17]. Decreased telomerase activity is associated with telomere irritation, inhibition of cell proliferative capacity through activation of p53-p21 or p16INK4a-Rb, and DNA Damage Response (DDR) consistent with features of cellular senescence [18–20]. In our study, the SIRT-1 activator significantly suppressed p16^{INK4a} expression in ADMA-exposed EPCs. This result suggests that activation of SIRT-1 through SIRT1720 is known to fight EPC aging due to ADMA induction. Recent studies have shown that eNOS-derived NO/SIRT1

cross-talk plays a role in maintaining mitochondrial biogenesis and may play a role in inhibiting SIRT-1-induced senescence [21, 22].

SIRT-1 exhibits an inhibitory effect on EPC aging by increasing telomerase activation via the PI3K-Akt signaling pathway. Inhibition of aging by activator SIRT-1 can protect EPC from dysfunction caused by pathological factors and increase the functional activity of EPC, which may be necessary for cell therapy applications [23]. Activating $p16^{INK4a}$ in response to stress results in progressive damage to several self-renewing tissues, including stem cells, while deletion of $p16^{INK4a}$ enhances cellular survival and regeneration potential. In line with the results of this study, the mechanism underlying the $p16^{INK4a}$-mediated cellular disruption in hematopoietic stem cells may be due to the upregulation of $p16^{INK4a}$ by chronic DNA damage due to progressive telomere dysfunction, which limits stem cell self-regeneration capacity [24].

4 Conclusion

In conclusion, our study successfully validated the role of ADMA in increasing $P16^{INK4a}$ expression in EPCs. In addition, this study has also proved that the SIRT-1 activator can inhibit senescence by decreasing $P16^{INK4a}$ expression in ADMA-exposed EPCs. The exposure indicates the protective effect of SIRT-1 against EPC senescence due to ADMA exposure.

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