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Intracellular p16^{INK4a} profile as targeted protein in human endothelial progenitor cells after chronic asymmetric dimethylarginine exposure

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Intracellular p16^{INK4a} Profile as Targeted Protein in Human Endothelial Progenitor Cells After Chronic Asymmetric Dimethylarginine Exposure

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Abstract. Downregulation of circulating EPCs survival affects the ability to maintain and repair the endothelium as a key feature in vascular homeostasis.The current study intends to describe the intracellular protein profile after chronic asymmetric dimethylarginine (ADMA) exposure using the immunofluorescence technique. Human EPCs as targeted cells were purified from peripheral blood mononuclear cells using Ficoll-based gradient centrifugation, cells were seeded on a culture plate and maintained in an endothelial growth medium (EGM) until 7 days. ADMA exposure was performed on the seventh day and incubate for 24 hours. The intensity of phosphorylated SIRT1 (pSIRT1) and p16^{INK4a} as targeted protein in ADMA treated cells were identified using a confocal laser scanning microscope. After 24 hours of ADMA exposure, intracellular protein expression in human EPCs was changed. Expression of p16^{INK4a} as cells cycle inhibitor protein was significantly higher in ADMA treated cells compared with control cells (p=0.000). Otherwise, the expression of pSIRT1 as a survival cells marker tends to decrease in ADMA treated cells compared with control (p=0.000). Our findings prove that elevated ADMA levels downregulating human EPCs viability.

Keywords: ADMA, EPCs, p16INK4a, pSIRT1

INTRODUCTION

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Elevation of serum ADMA has been reported as the risk factor for cardiovascular disease by accelerating endothelial dysfunction, which also induced EPCs dysfunction [5]. The mechanism of EPCs dysfunction due to ADMA remains unclear, but a previous study report that apoptosis EPCs by ADMA occurred after endoplasmic reticulum stress sensor protein activation [6]. Endogenous ROS formation by ADMA modulated various intracellular

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processes as well as senescence-associated proliferation arrest [5]. As cells cycle regulator, p16INK4a prevents G1 to S phase progression through inhibition of CDK4/6 kinases [5, 7]. The clearance of p16INK4a expressing cells attenuates age-associated phenotype and improves healthy cells lifespan [8].

Correspondingly, it was shown that transcriptional regulation p16INK4a is instead dependent on the proper function of SIRT1. A growing body of evidence suggests that SIRT1 has a beneficial effect to prevent endothelial cells become senescence [9]. But it is unclear whether SIRT1 activity has the same effect in human EPCs. Therefore, in the current study, we intend to describe: first, whether chronic ADMA exposure leads to cell cycle arrest by intracellular p16INK4a enhancement, second, whether the elevation of p16 after ADMA occurs after match pSIRT1 reduction.

EXPERIMENTAL DETAIL

Cell Isolation and Culture

5 mL heparinized peripheral blood was acquired from a healthy subject after informed consent in conformity with the local ethics committee. EPCs were isolated from PBMNCs using Lymphoperm^{TM} density gradient solution (density 1.077; Axis-shield, France) according to the manufacturer's protocol. The procedures for the purification of EPCs from human PBMNCs have been describing elsewhere.[10] PBMNC were seeded on the culture dishes with human fibronectin-coated (Clonetech, Switzerland) and maintained in EGM^{TM}-MV2 medium BulletKit^{TM} (Cat: CC-3202, Lonza, Swiss) supplemented with 15% fetal bovine serum (FBS), 1% L-glutamine, and 1% Penicillin/streptomycin (Gibco) in a 5% CO_2 chamber at 37 ^{0}C until seven days to get a spindle shape morphology [1, 11, 12].

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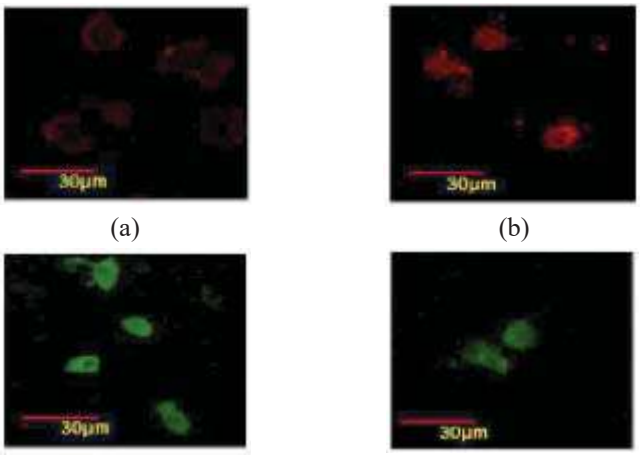
Cells sample preparation done as well as p16^{INK4a}staining procedure. EPCs stained with anti-human SIRT-1 (pT530) (JJ206-6) as primary antibodies were applied overnight at 4 ^{0}C. After washed, the fixated cells were stained with Goat anti-rabbit IgG H&L (Alexa Fluor 488) (Ab150077, Abcam) according to the manufacturer's instruction.

Data Analysis

All trials were executed in triplicates. Data are provided as mean ± standard deviation (SD). Mann-Whitney tests were performed to compare means in each group. The statistical interpretation was decided at $p<0.01$. STATA (Stata Corporation, College Station, TX, version 14 for MacBook Air) was used for statistical analysis.



(a) (b)

(c) (d)

FIGURE 1. The intensity of intracellular protein profile on human EPCs: (a) intracellular $p16^{INK4a}$ profile in control cells; (b) Intracellular $p16^{INK4a}$ profile in ADMA treated cells; (c) Intracellular pSIRT1 profile in control cells; (d) Intracellular pSIRT1 in DMA treated cells. Scale bar represent 30µm. The experiment was performed in triplicate and similar outcome were obtained each time.

RESULT AND DISCUSSION

Intracellular $p16^{INK4a}$ profile after chronic ADMA exposure

We first examine whether $p16^{INK4a}$ tends to increase in human EPCs after chronic ADMA exposure. To characterize the $p16^{INK4a}$ profile, an immunofluorescence technique was performed. The fluorescence intensity of $p16^{INK4a}$ in treated cells and control are presented in Fig. 1. Furthermore, chronic ADMA exposure to human EPCs leads to the elevation of $p16^{INK4a}$ significantly compared to control cells ($p=0.000$) as presented in Table 1. It means that the cell cycle arrest was activated as a stress response by increasing the CDK inhibitor $p16^{INK4a}$ after chronic ADMA exposure. Our finding possibly explained that upregulation of $p16^{INK4a}$ is a cell compensation if proper cell division cannot occur due to the presence of DNA damage [9,13,14]. Cell cycle controlling proteins, such as $p16^{INK4a}$ and p21 have an important assignment to encourage senescence features in response to DNA damage, on the other hand, SIRT1 can positively regulate DNA repair.[15]

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Intracellular protein profile	Control	ADMA treated cells	P.value
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A novel targeted approach to prevent EPCs dysfunction is needed. Our findings show that ADMA leads to senescence-associated upregulation of $p16^{INK4a}$ as diverse stress. Therefore, Inhibition of $p16^{INK4a}$ may have a promising impact to reduce the negative impact of ADMA exposure on human EPCs. Basically, $p16^{INK4a}$ is also capable of triggering tumor suppressor pathways which inhibit cells proliferation trough the direct inhibition of cells cycle progression [16,17]. Previous research identified that histone deacetylases 3 and 4 inhibit the activity of the $p16^{INK4a}$ promoter trough Yin Yang 1 and zinc-binding protein-39 as downstream transcription factor of SIRT1 [18].

To further elucidate whether elevation $p16^{INK4a}$ profile after ADMA exposure is related to the downregulation of pSIRT1, we investigate the fluorescence intensity of intracellular pSIRT1.

Intracellular pSIRT1 Profile After Chronic ADMA Exposure

The regulation of mitochondrial homeostasis by SIRT1 is a key factor to prevent telomere erosion that irreversible stops cell cycle progression [19-21]. Recent development elucidated that the downregulation of SIRT1 correlated with an increase in the oxidative stress-related cellular event, inflammation, and telomere damage induces replicative senescnece by enhancement of acetylated p53 [22]. Based on Table 1, our results suggest that pSIRT1 tends to decrease significantly after chronic ADMA exposure, this finding indicates the significance elevation of $p16^{INK4a}$ in response to ADMA exposure related with the presence of telomere attrition. Because it also has been reported that $p16^{INK4a}$ contribute to the p53-independent in response to telomere dysfunction [23].

The reduction of pSIRT1 in this study exhibit that SIRT1 repressed by tumor protein p53, the most important tumor suppressor as well as describe before [24, 25]. When ADMA accumulation occurs, expression p53 tend to elevate as a result of protein arginine methyltransferase 1 (PRMT1) silencing which has implicated in DNA damage response [26]. Stable low level of PRMT1 showed reduced growth rate and cause cell cycle arrest [27].

CONCLUSION

This study suggests that the intracellular pSIRT1 tends to decline if human EPCs were exposed to ADMA continuously. Subsequently, upregulation of p16INK4a after ADMA exposure may reflected DNA damage present which leads to cell cycle arrest to avoid cell division with damaged DNA. Therefore, Inhibition of p16INK4a may have a promising impact to reduce the negative effect of ADMA in human EPCs. This finding suggests that reversal of EPCs dysfunction could therefore potentially prevent the cardiovascular disease progression by downregulating p16INK4a as targeted protein.

ACKNOWLEDGMENTS

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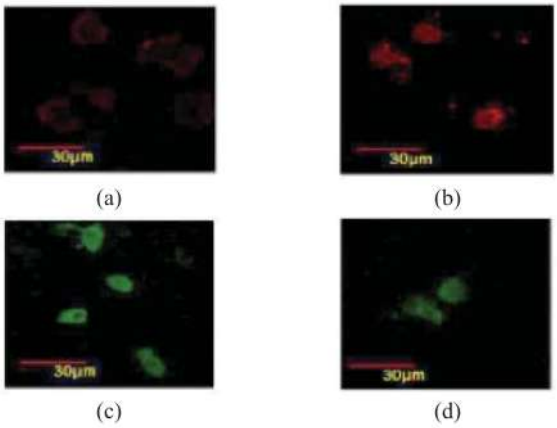

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