Small Chain Fatty Acid Phenylbutyric Acid Alleviated Inflammation-Induced Endoplasmic Reticulum Stress in Endothelial Cells

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Abstract- Endothelial cells (EC) have dynamic properties and high plasticity in response to microenvironmental change. A proinflammatory cytokine such as tumor necrotizing factor-α (TNF-α) can induce EC phenotype shift to osteoinduction properties by releasing a potent osteogenic cytokine, namely bone morphogenetic protein 2 (BMP2). Normally BMP2 acts as an osteoblast stimulating factor in bone and cartilage tissue. BMP2 activation in vascular tissue will invite osteoblast recruitment and mineralization and generated pathological vascular stiffening and calcification. Recently, endoplasmic reticulum stress (ERS) has been emerging as a new target therapy in many vascular diseases such as vascular stiffening and calcification. Some short-chain fatty acid like 4-phenyl butyric acid has been shown had anti-ERS properties. However, the role of 4-phenyl butyric acid in BMP2 inhibition in endothelial cells is still poorly understood. Hence, we investigated the role of 4-phenyl butyric acid in inflammation-induced BMP2 expression in human vein derived endothelial cells. Endothelial cells obtained from a baby born umbilical vein were cultured and pre-treated with TNF-α (5 ng/ml) as inflammation precondition. Multiple doses of 4-phenyl butyrate acid (4-PBA) 1 nM/mL, 2 nM/mL, and 3 nM/m were used as ERS inhibitors. The expression of two ERS biomarkers, glucose-related protein-8 (GRP78) and activating transcription factor-6 (ATF6), were measured. Statistical analysis was done using one-way ANOVA and Kruskal Wallis tests, and $P<0.01$ considered as significant. 4-PBA decrease luminal BMP2 at dose one nM/L, GRP78 at dose 1 nM/L, and translocated ATF6 expression at dose 1 nM/L in endothelial culture dose-dependently. Short-chain fatty acid 4-phenylbutyrate acid decreases luminal ERS marker GRP78 and translocated ATF6 expression in endothelial culture. ERS has a role in osteoinductive phenotype shifting in inflammation endothelial cells, which was the novelty of this research. Further research needs to elucidate ERS inhibition in *in vivo* experiment

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Keywords: Bone morphogenetic protein-2; Endoplasmic reticulum stress; Endothelial cells; Small chain fatty acid; Tumor necrosis factor-α

Introduction

Endothelial cells (EC) is one of the body's cells that have dynamic properties and has high plasticity (1,2,3). Biochemical and hemodynamic changes of blood flow require high adaptive endothelial cells to maintain homeostasis of blood flow. Physiologically, this plasticity was required in the process of normal growth and development of early life embryogenic formation. However, in certain pathological processes, this plasticity leads to the phenotypic shift of normal EC to contribute to disease progression (4).

It has been well known that high vascular stiffness has a fundamental role in hypertension pathomechanism (5). Among its molecular pathways, vascular calcification (VC) has gained as the most important factor that contributed to vascular stiffness (6,7). VC is a pathological condition characterized by the deposition of the mineral crystals of calcium in the tunica media of the major arteries, which leads to an increase in the blood vessel stiffness (8). Stimulation of osteoinductive cytokine such as bone morphogenetic protein 2 (BMP2) will induce vascular smooth muscle cells (VSMC) to change their phenotype into osteogenic properties and

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invite more deposition of hydroxyapatite crystals in the matrix (9).

TNF-α has been well-known as a potent proinflammatory cytokine. As a pleiotropic cytokine, it has been known that TNF-α had an important role in the osteogenesis process. Dependent on its micro-environment, in osteal tissue formation, it will accelerate osteoclast activity which leads to bone resorption and the final stage of osteoporosis (10,11). In extra osteal tissue, such as vascular tissue, it promotes mineral deposition lead to its calcification (12,13). This will lead to a decrease in vascular elasticity and increase vascular stiffness and contributed to systolic hypertension (14,15). It has been widely reported that TNF-α promotes osteogenic differentiation shift in human vascular smooth muscle (VSMC) (16). TNF-α has a pivotal role to change phenotype shift vascular mesenchymal tissue into osteogenic phenotype (17,18). Moreover, endothelial cells are one of TNF-α primary target organ-damaged leads to its dysfunction and contribute to vascular calcification too (19). Some articles report TNF-alpha-exposed endothelial cells to undergo osteoinductive shifts (20). Osteoinductive phenotype shift will lead the endothelial cell to secreted bone morphogenetic protein 2 (BMP2) which is a strong bone inductor in osteoblast and bone formation (21). Despite even the roles of TNF-α in VSMC and MC are well documented, yet their roles in endothelial cells are less defined. It is still poorly understood how TNF-α regulates endothelial BMP-2 expression. Hence, this study aims to investigate whether TNF-α regulates BMP-2 expression dose and time-dependently.

In endothelial cells, stimulation of proinflammatory cytokines such as TNF-α will stimulate its phenotype shift to be more osteoinductive. Furthermore, this endothelial phenotype shifts initiates VSMC loss of its contractility and became an osteogenic lineage. Osteoinductive endothelial cells will increase the expression of potent bone tissue inductor, bone morphogenetic protein 2 (BMP2) which changes the natural properties of VSMC in the cells lining the tunica. Unfortunately, this osteoinductive mechanism of endothelial cells is still not widely known.

On the other hand, a cellular level mechanism that recently emerged and became a more attractive research area is what is known as the endoplasmic reticulum stress (ERS). ERS defines as endoplasmic reticulum (ER) dysfunction due to ER's inability to increase its capacity in the folding process of the protein. Base on it, it was interesting to dig deeper with the question of whether ERS participates underlying nature of the process of change towards osteoinductive endothelial cells. With that question, we hypothesized that participate in ERS processes underlying the process of the changing nature of endothelial cells towards osteoinductive. It is hoped this knowledge will fill the knowledge gap to inhibit the processes of advanced due to the VK.

Materials and Methods

The study type was experimental in-vitro with case-control design and was approved by the Human Ethical Committee of Brawijaya University, Malang, Indonesia (141/EC/KEPK/S3/05/2016). Human umbilical vein endothelial cells (HUVECs) were obtained from donors with written informed consent. Briefly, endothelial cells isolated by 0.05% collagenase type I digestion (from Clostridium histolyticum, Worthington, Lakewood, NJ, I.U.B catalog .3.4.24.3) for 15 minutes. The medium then changed to remove blood components and non-adherent cells. For primary culture, the adherent cells were plated into 25 cm^2 Falcon flasks in medium RPMI-1640 containing 25 mM HEPES and L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 10% heat-inactivated fetal calf serum (FCS), and 10% new-born calf serum (NBCS). The cells were placed in a 5% CO2 incubator at 37° C for 3 hours to allow cell adhesion. After cells reached 70-80% confluence, EC was enzymatically detached using 0.05% trypsin/ 0.02% EDTA and sub-cultured at a 1:3 split ratio in the above EC cell growth medium containing, in addition, 15 mg/ml endothelial cell growth supplement (Sigma Chemical Co., UK) and 50 U/ml heparin (Leo Laboratories Limited, UK). Cells then plated at density 3000-4000 cells/cm3 on 24 well plate cell culture dishes (Falcon; BD Biosciences, New Jersey) in M199 medium with 20% Fetal Bovine Serum, 100 mg/ml pen-strep, 0.1 mg/ml heparin, and 0.05 mg/ml EGF. Cells culture was then incubated at 37° C in a 5% CO_2 humidified incubator. At 70-80% confluency, four groups were exposed to TNF-α 5 ng/ml (Biolegend, California), and three groups were exposed to 4-PBA (Bioss, Beijing, China) at three different doses 1, 2, and 3 mM/L for 8 hours. Cells were fixed with methanol 5% and immunostained with BMP-2 antibody (Bioss, Beijing, China), GRP78 (Bioss, Beijing, China), and ATF6 (Bioss, Beijing, China). Data were analyzed by one-way ANOVA, and the difference between groups was analyzed by post hoc LSD comparison test. Data are expressed as mean±standard error of the mean (SEM). *P* less than 0.05 were considered significant statistically.

Results

GRP-78

In this study, we show that TNF-α increases endothelial GRP78 expression in the positive control (B) and minimally expressed in the negative control (A). As shown in Figure 1, 4-PBA treatment had decrease TNF-induced-GRP78 expression significantly in a dose-dependent manner. Positive stained of GRP78 cells in B (control positive) show decrease gradually after 4-PBA treatment at dose 1, 2, and 3 nM/L. The minimally GRP78 expression has been shown in 4-PBA 3 mM/L group and had no difference statistically compared to the negative control (A).

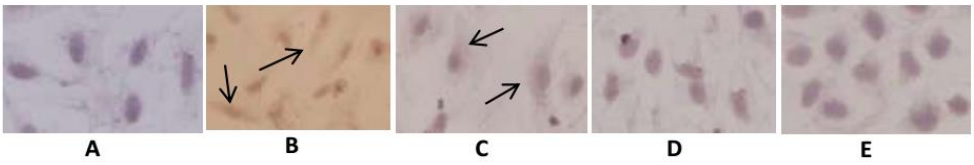

Figure 1. Immunostaining of GRP78 has been shown different results between TNF only (B) and PBA treatment group (CDE). Dose 3 nM/L of PBA € has a minimum expression of nuclear ATF6 compare to a lower dose (C and D). Arrows show GRP78 positive stained cells (magnification 400X)

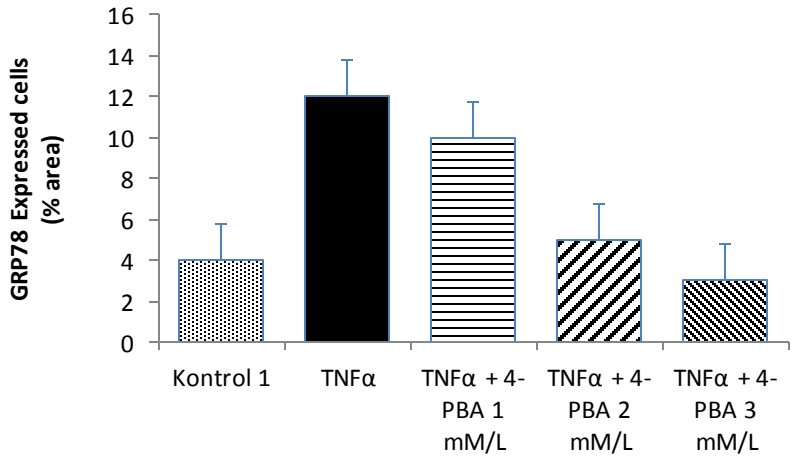

Table 1. GRP78 decreased dose-dependently in endothelial culture after PBA treatment groups

ATF6

TNF-α increases endothelial nuclear ATF6 expression in the positive control (B) and minimally expressed in the negative control. As shown in Figure 2, PBA treatment had decrease ATF6 dose-dependently significantly. As seen in immunocytochemistry results, many positive ATF6 stained cells in control positive show a decrease gradually after PBA treatment. The minimally ATF6 expression is shown in PBA 3 mM/L and not significant statistically compared to a negative control without TNF-α treatment.

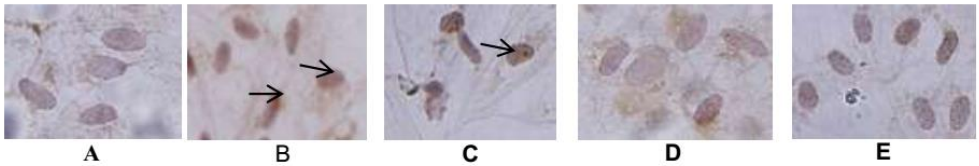

Figure 2. Immunostaining of nuclear ATF6 has been shown different results between TNF only (B) and PBA treatment group (CDE). Dose 3 nM/L of PBA € has a minimum expression of nuclear ATF6 compare to a lower dose (C and D). Arrows show ATF6 positive stained cells (magnification 400X)

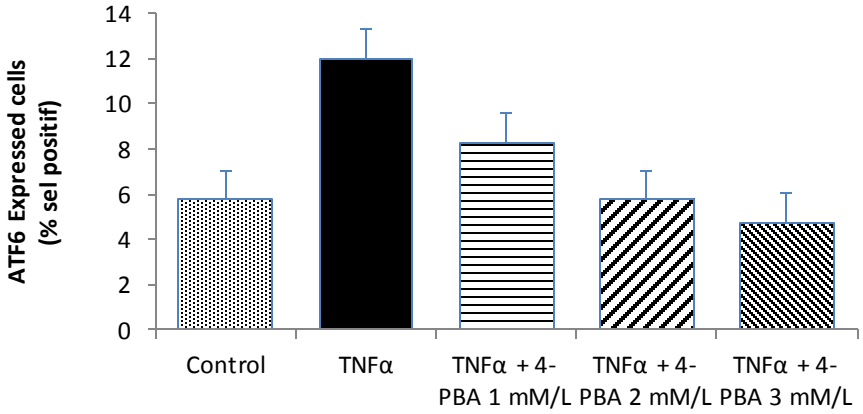

Table 2. ATF6 decreased dose-dependently in endothelial culture after PBA treatment groups

BMP 2

TNF-α increases endothelial BMP2 expression in positive control and minimally expressed in the negative control. As shown in Figure 3, PBA treatment has to decrease BMP2 dose-dependently significantly. As seen in immunocytochemistry results, many positive BMP2 stained cells in control positive show a decrease gradually after PBA treatment. The minimally BMP2 expression is shown in PBA 3 mM/L and not significant statistically compared to a negative control without TNF-α treatment.

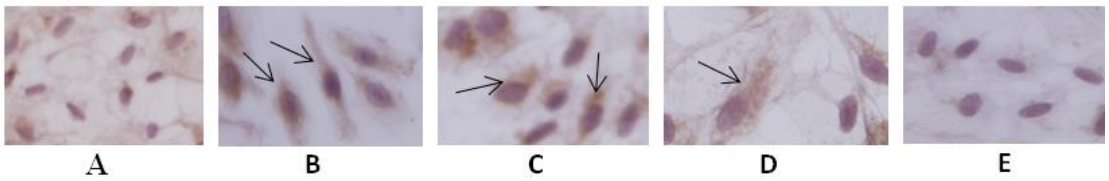

Figure 3. Immunostaining of BMP2 has been shown different results between TNF only (B) and PBA treatment group (CDE). Dose 3 nM/L of PBA € has a minimum expression of BMP2 compare to lower doses (C and D). Arrows show BMP2 positive stained cells (magnification 400X)

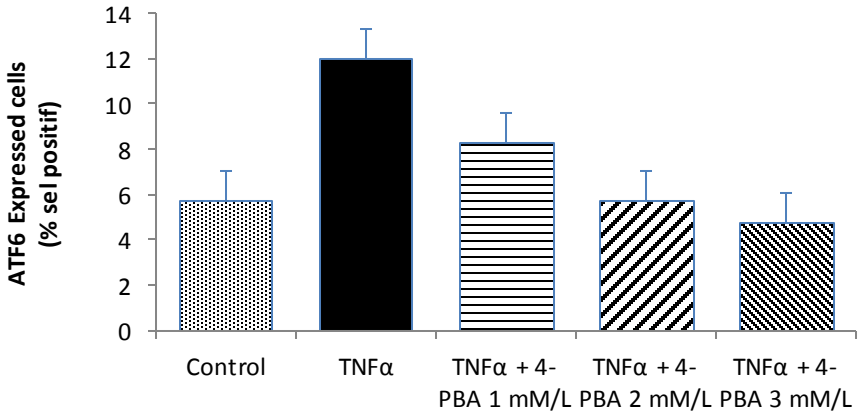

Table 3. BMP2 expression decreased dose-dependently in endothelial culture after PBA treatment groups

Discussion

The osteoinductive changes of endothelial cells are one of the properties of endothelial cell plasticity (22). In physiological conditions, this property has a vital position to maintain homeostasis due to changes

resulting from vascular injury. At one point, however, the change of plasticity is a crucial factor in the pathological process of disease (4). In the process of vascular calcification, the vessel's consistency change as a response to the hemodynamic flow of blood (23). Triggered by chronic, low-intensity chronic inflammation, the endothelial cells change in properties with the expression of calcium initiator proteins such as BMP2 (24). Moreover, it will change the nature of endothelial cells, which will shift its phenotype to osteoinductive properties and have a pivotal role in initiating vascular stiffness pathomechanism (25).

In this study, we took two of ERS mediators, ATF6 and GRP78, to see their expression in certain exposure. We used TNF-α proinflammatory cytokines to induce inflammation milleu of endothelial cells. TNF-α was used with concentrations of 5 ng/ml for eight hours of exposure time (26). Such a dose more than this, 20 ng/ml, has proven to induced BMP2 in chondrocyte tissue but not in endothelial cells (27). GRP78 parameters were used to measure intra-luminal ERS mediators and ATF6 parameters to measure intra-cytoplasmic ERS mediators (28,29). Short chain fatty acid 4-PBA is used as selective and potent ERS inhibitors (30).

For the first time, we present evidence of the involvement of ERS in the process of the osteoinductive shift of EC, and it can be inhibited by 4-PBA. This change has been shown by the expression of BMP-2 osteogenic protein in endothelial cells. BMP-2 in endothelial cells can be induced by stimulation of TNF-α proinflammatory cytokines (9,31,32). This will disrupt endothelial function and lead endothelial turn to osteogenic lineage (33,34). However, some papers report that this osteogenic shifting of endothelial cells has self-protective action rather than a pathologic process (35).

The mode of action PBA is a chemical chaperon that assists the biological chaperones in the RE lumen (36). PBA is a short-chain fatty acid that has long been used as an ammonia scavenger in urea cycle disorder (urea cycle disorder). As a chemical chaperon, PBA works by reversing the process of mislocalization and protein aggregation that occurs in some diseases (37). In other words, the PBA acts as an adjunct agent of the biological chaperon molecules present in the RE lumen. This activity could be protected EC from certain apoptotic mechanisms induced by inflammation (38). As indicated by the above results, the provision of PBA significantly reduced the expression of GRP78 compared with the positive control group at doses of 1, 2, and 3 mM/l. Others researcher has found that PBA more than our dose, 5nM/l has significant effect to decrease GRP78 (39). This level of GRP78 decreased equal to the negative control level was found at doses of 2 and 3 mM/L as indicated by the LSD posthoc test statistically. Furthermore, as shown in the graphic figure, the administration of PBA decreases the expression of ATF6 nuclei at doses of 1, 2 and 3 mM/L. This corresponds to the results obtained by Zhang showing that PBA has the effect of lowering ATF6 expression although this report is still limited to renal epithelial cells from rats (30). In the third experiment, as shown in the above results, PBA administration decreased the expression of the target protein BMP-2 endothelial cells significantly at doses of 2 and 3 mM/L. At a dose of 2 mM/L, BMP2 level statistically equal with as negative control. Interestingly, higher doses of PBA decreased BMP2 lower than negative controls. These results, although still need to be confirmed again in future studies, need not be confused because BMP2 is an important protein and overexpressed in the normal growth process (40). Ultimately, these results suggest that BMP-2 expression as a result of TNF-α exposure may be inhibited by PBA as a selective inhibitor of ERS.

Moreover, the Pearson correlation test showed that the GRP78 decrease had a strong correlation to the decrease of BMP2 ($r=0.887$, $P<0.00$). Additionally, the decrease in ATF6 also has a strong correlative relationship with the decreased of BMP2. ($R=0.783$, $P<0.00$). A strong correlative relationship was also obtained between GRP78 and ATF6 ($r=0.883$, $P<0.00$). This correlative relationship indicates that osteoinductive changes in endothelial cells shown by BMP2 expression have a causal relationship mediates by ERS.

Inhibition of ERS is reported to have an inhibitory effect on the pathomechanism of cardiovascular disease especially in the hardening of large blood vessels (41,42). In the process of atherosclerosis, ERS also can not be underestimated again its role in initiating the occurrence of disease (43). Nevertheless, as far as our knowledge, no study has been conducted before concerning osteoinductive shift endothelial cell-related in endoplasmic reticulum stress. Although these results are still based on a small portion of the ERS markers, at least our result has provided a new leap of ERS involvement in the process of changing the osteoinductive properties of endothelial cells caused by vascular inflammation. From the result, we know that PBA as small fatty acid has a remarkable effect in inhibiting the osteoinductive shift of endothelial cells.

As small chain fatty acid, phenylbutyrate acid decrease luminal ERS marker GRP78 and translocated ATF6 expression in endothelial culture. GRP78 and translocated ATF6 correlate with BMP2 expression and is dose-dependent mainly at 4-PBA dose 2 and 3 nM/L. From the result, it has been concluded that small fatty acid PBA can inhibit endoplasmic reticulum stress-induced-osteoinductive phenotype shifting in inflammation endothelial cells. Small chain fatty acid has a pivotal role in inhibiting endothelial dysfunction caused by vascular inflammation.

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