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Original Article

Pre-B-cell colony enhancing factor (PBEF) increases endothelial permeability in hypoxia/re-oxygenation model

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Abstract: Objective: This study aims to explore the relationship between PBEF and VEGF and p-MLC and the mechanism of PBEF increasing permeability of endothelial cells in hypoxia/re-oxygenation. Methods: Hypoxia/re-oxygenation model was established and PBEF siRNA was synthesized. According to the different HUVEC treatment, it can be divided into normal control group, PBEF siRNA group; hypoxia (20 hours) and re-oxygenation (3 h) group, hypoxia (20 h) and re-oxygenation (6 h) group, hypoxia (20 h) and re-oxygenation (9 hours) group, hypoxia (20 h)/re-oxygenation (12 h). The expressions of PBEF, VEGF and p-MLC were tested by RT-PCR and Western blot. Results: The mRNA and protein expression of PBEF in PBEF siRNA group were significantly lower compared to liposome group and the negative controls ($P < 0.05$). The expression of PBEF protein in hypoxia/re-oxygenation group was significantly higher than the normal control group. It increased in the 3 h of re-oxygenation group, peaked at 9 h, until 12 h started to decline ($P < 0.05$). When the PBEF gene was knockdown, the expression of VEGF and p-MLC in hypoxia and re-oxygenation are significantly lower. Conclusions: PBEF siRNA can effectively inhibit the expression of PBEF in endothelial cells. The expression of PBEF, VEGF and p-MLC were significantly higher in endothelial cell after Hypoxia/re-oxygenation. PBEF may change the permeability of endothelial cells by regulating the expression of VEGF and the phosphorylation of MLC.

Keywords: Pre-B-cell colony enhancing factor (PBEF), RNA interference, hypoxia/reoxygenation, VEGF, p-MLC

Introduction

The pulmonary dysfunction after cardiopulmonary bypass (CPB) is one of the most common clinical problems and the reason for most important complications and high death rate after CPB [1, 2]. The mechanism of lung injury induced by CPB is very complicated and is not very clear. In general, it is thought to be related to the systemic inflammation and the occurrence of ischemia reperfusion injury [2-4]. It is particularly important to research on the mechanism of lung injury after CPB and how to protect the lung. In recent years, many scholars carried out a series of experimental and clinical studies on the mechanisms of lung injury and lung protection measures after CPB [4-7].

Pre-B-cell colony-enhancing factor (PBEF), also named Visfatin or Nampt, is a biochemical and genetic marker in acute lung injury (ALI). Overexpression of PBEF can augment the expression of inflammatory cytokines and dysregulate pulmonary cell barrier function, whereas inhibition of PBEF expression by its cognate siRNA has the opposite effects. It plays an important role in various acute and chronic inflammations [8-10].

Vascular endothelial growth factor (VEGF) is one of the most potent proangiogenic growth factors, it participates in angiogenesis and increases vascular permeability [11, 12]. Various cytokines involved in the regulation of VEGF gene expression. Whether increased PBEF expression resulted in increased endothelial permeability through regulating the expression of VEGF? In this study, we explored the relationship between the expression of PBEF, VEGF and Phosphorylation-myosin light

chain kinase (p-MLC) in vitro, which could lay the foundation for the research on the regulation mechanism of increased vascular endothelial permeability induced by PBEF after CPB.

Materials and methods

Cell culture

The HUVEC cells were cultured with DMEM containing 10% fetal bovine serum at 37°C with 5% CO_2.

RNA interference

The sequences for PBEF siRNA were as follows: sense sequence: 5'-CCACCCAACACAAGCAAAGUUUAUU-3'; antisense sequence: 5'-AAUAAAGUUUGGUUGUGUUGGGUGG-3'. The sequences for control were as follows: sense sequence: 5'-UUCUCCGAACGUGUCACGUTT-3'; antisense sequence: 5'-ACGUGACACGUUCGGAGATT-3'. They were used to transfect into HUVEC cells with lipofectamine 2000 kit according to the manual.

The effectiveness of PBEF siRNA sequences was detected using RT-PCR method

Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manual protocol. Total RNA was subjected to reverse transcription using PrimeScript RT reagent Kit according to the manual. Real-time PCR were performed using SYNBR Green PCR Master Mix (Qigen). β-actin gene was used as an internal control for normalization of RNA quantity. The primers for PBEF gene were as follows: F: 5'-AAGCTTTTTAGGGCCCTTTG-3'; R: 5'-AGGCCATGTTTTATTTGCTGACAAA-3'; the primers for β-actin gene were as follows: F: 5'-TAAGAAGCTGCTGTGCTACG-3'; R: 5'-GACTCGTCATACTCCTGCTT-3'.

The effectiveness of PBEF siRNA sequences was detected using Western blotting method

Total proteins were extracted after interference for 48 h. The protein content was determined using BCA kit and analyzed with SDS-PAGE electrophoresis. Then it was electrotransferred to the PVDF membrane. The membrane containing the proteins was used for immunoblotting with required antibodies for PBEF and β-actin. They were blocked with 5% non-fat milk in TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween-20) for 2 h at room temperature, then incubated with the primary antibodies for 2 h at room temperature. They were incubated with secondary antibodies conjugated with horseradish peroxidase at room temperature for 1-2 h after that. The membrane was developed and fixed and images were analyzed using Quantity One software.

The model of hypoxia and reoxygenation

Hypoxia and reoxygenation model was established when the cells were in good condition and the fusion rate was about 80%. The medium was changed to hypoxic liquid and the culture dish was put into the sterilized hypoxic box. 5% CO_2 and 95% N_2 was charged into the hypoxic box and the concentration of oxygen in hypoxic box reached less than 1% after more than half an hour. Then the box was sealed and cultured in 37°C incubator for 20 hours. After that they were cultured with normal medium at 37°C for reoxygenation for 3 h, 6 h, 9 h and 12 h respectively to select the time of reoxygenation. The expression levels of PBEF were detected by Western blotting method.

Detection of the expression of VEGF and p-MLC

The cells were divided into 4 groups. A: Hypoxia/reoxygenation and empty liposomes group; B: Hypoxia/reoxygenation and negative control group; C: Hypoxia/reoxygenation and PBEF-siRNA group; D: empty liposomes group. The expression levels of VEGF and p-MLC were detected with Western blotting method.

Statistical analysis

Values are presented with mean ± SD. All data analysis was performed with SPSS 17.0 software. The data were analyzed with ANOVA. $P < 0.05$ was considered to be statistical significance.

Results

The effectiveness of PBEF siRNA sequences

The transfection efficiency observed by Fluorescence microscope was shown in **Figure 1**. The effectiveness of PBEF siRNA sequences were detected with RT-PCR and Western blotting methods. Using β-actin as control, the relative

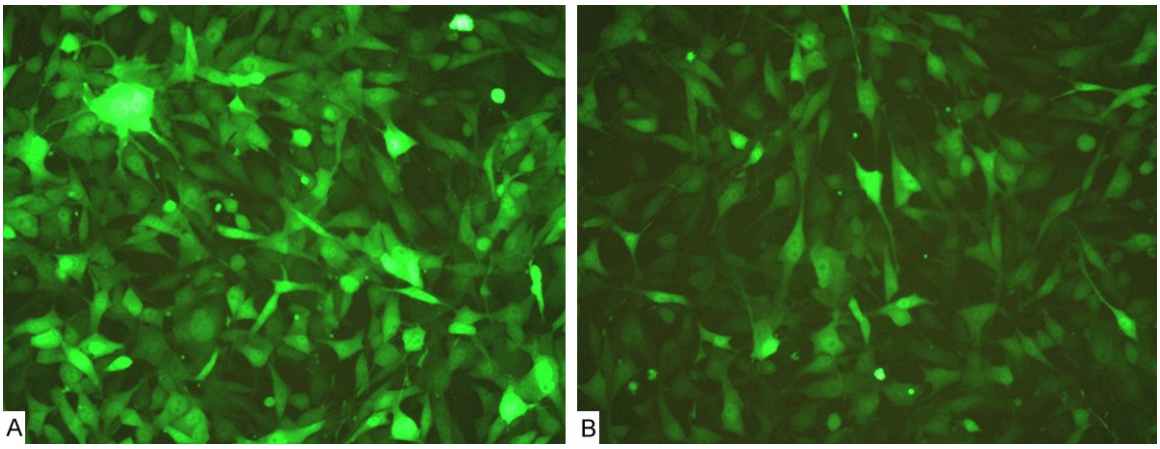

Figure 1. The transfection efficiency observed by Fluorescence microscope. A: Control group; B: PBEF siRNA group.

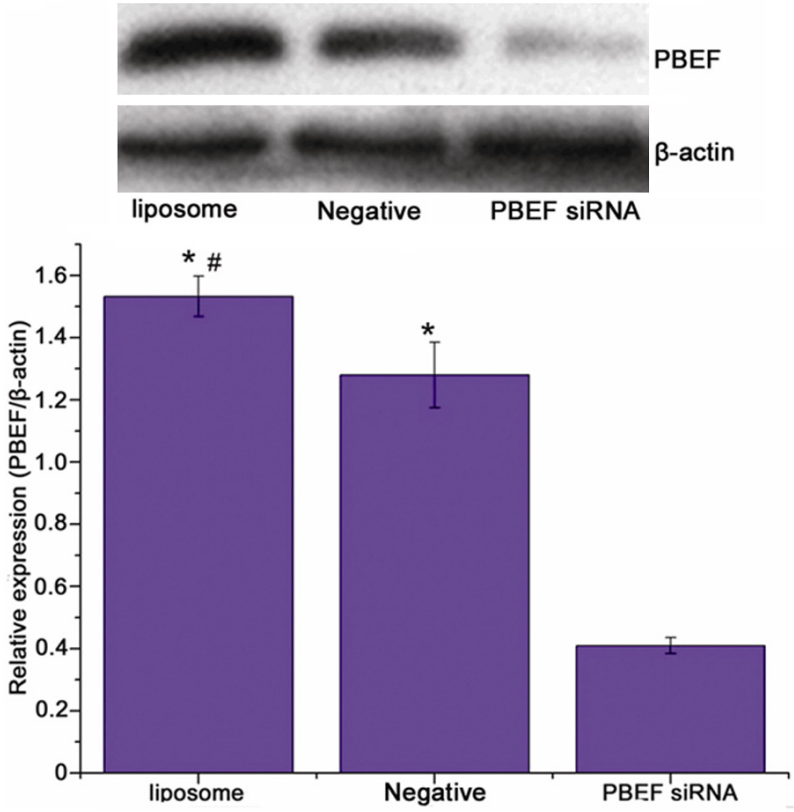

Figure 2. Western blotting results of the effectiveness of PBEF siRNA sequences. *P < 0.05 vs PBEF siRNA group; #P > 0.05 vs negative control group.

expression level of PBEF in PBEF siRNA group was significantly lower than that of liposome group and negative control group (P < 0.05), there was no difference between liposome group and negative control group (P > 0.05). The results of Western blotting were shown in **Figure 2**.

The expression of PBEF in the model of hypoxia and re-oxygenation

As shown in **Figure 3**, compared with the normal control group, the expression of PBEF was significantly increased in hypoxia and re-oxygenation group. It began to increase after re-oxygenation for 3 h (2.34 ± 0.48) and reached the peak value after re-oxygenation for 9 h (3.43 ± 0.25). It began to decrease after re-oxygenation for 12 h (2.26 ± 0.07) (P < 0.05).

The expression of VEGF in HUVEC cells

The expression levels of VEGF in group A (0.84 ± 0.03) and group B (0.9 ± 0.1) were significantly higher than that of group D (0.49 ± 0.07) (P < 0.05), which suggested that hypoxia/re-oxygenation induced expression of VEGF in HUVEC cells, thus increased the cellular permeability. There was no difference between group A and group B (P > 0.05). The expression level of VEGF in group C (0.29 ± 0.02) was significantly lower than that of group A and group B (P < 0.05), which suggested that PBEF siRNA had the protective cel-

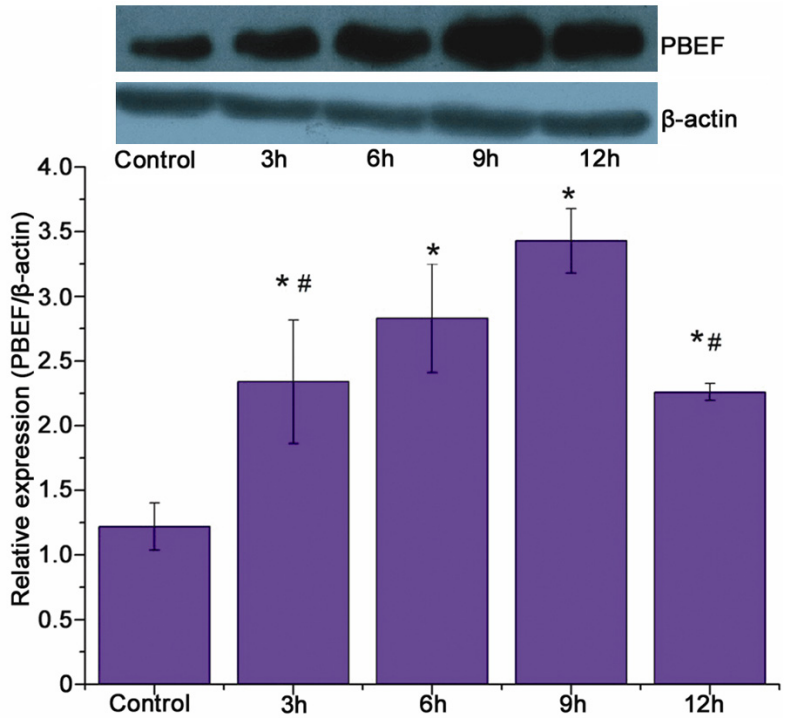

Figure 3. The expression levels of PBEF in the model of hypoxia and reoxygenation at different time point. *P < 0.05 vs control group; #P < 0.05 vs reoxygenation for 9 h group.

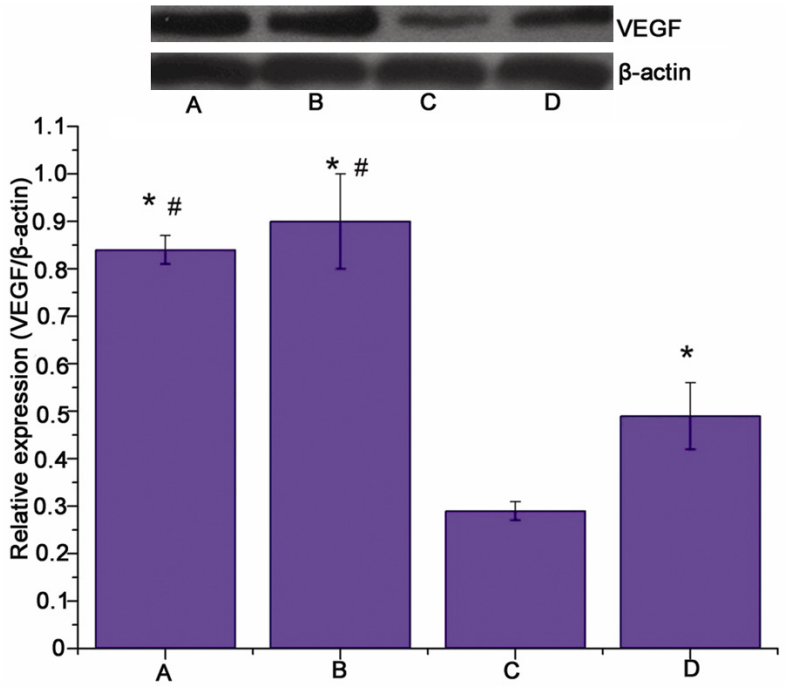

Figure 4. The expression of VEGF in HUVEC cells. A: Hypoxia/reoxygenation and empty liposomes group; B: Hypoxia/reoxygenation and negative control group; C: Hypoxia/reoxygenation and PBEFsiRNA group; D: empty liposomes group. *P < 0.05 vs group C; #P < 0.05 vs group D.

lular barrier function (**Figure 4**).

The expression of p-MLC in HUVEC cells

The expression levels of p-MLC in group A (0.71 ± 0.07) and group B (0.66 ± 0.06) were significantly higher than that of group D (0.23 ± 0.01) (P < 0.05), which suggested that PBEF siRNA decreased the phosphorylation of MLC in HUVEC cells after hypoxia/re-oxygenation. The expression of p-MLC in group C (0.36 ± 0.09) was significantly lower than that of group A and group B (P < 0.05), which suggested that the increased cellular permeability after hypoxia/re-oxygenation by PBEF was probably through the phosphorylation of MLC (**Figure 5**).

Discussion

The RNA interference (RNAi) was first discovered by observations of transcriptional inhibition by antisense RNA expressed in transgenic plants [13], which is a biological process that RNA molecules inhibit gene expression. Small interfering RNA (siRNA) is central to RNAi. The siRNA can bind to specific messenger RNA (mRNA) molecules and either increase or decrease their activity. It is a valuable research tool and often used to study the function of genes in cell culture and in model organisms [14-17].

PBEF is a highly conserved, 52-kDa protein with a number of discrete biological actions. It can act like a cytokine and in monocytes

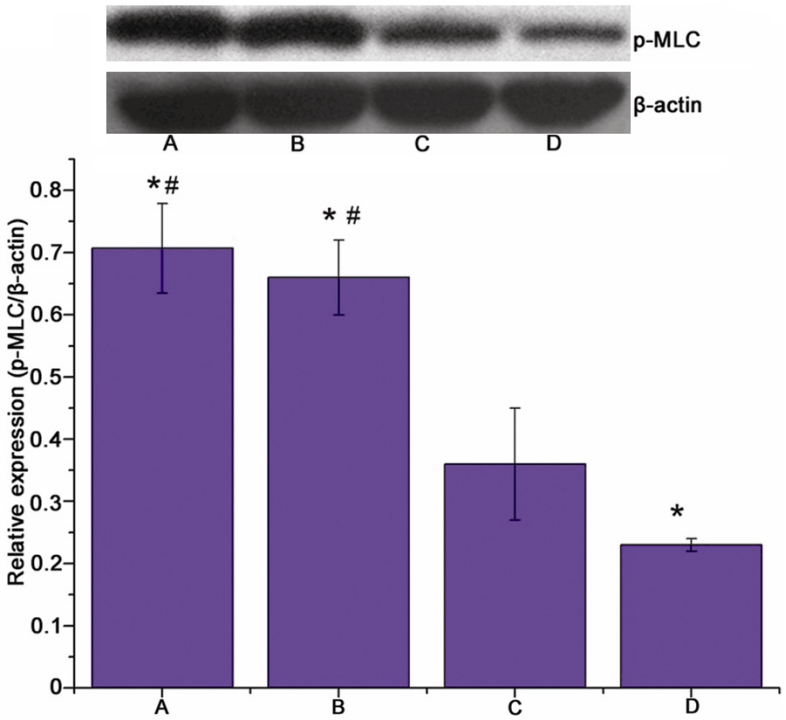

Figure 5. The expression of p-MLC in HUVEC cells. A: Hypoxia/reoxygenation and empty liposomes group; B: Hypoxia/reoxygenation and negative control group; C: Hypoxia/reoxygenation and PBEFsiRNA group; D: empty liposomes group. *P < 0.05 vs group C; #P < 0.05 vs group D.

and has been shown to stimulate expression of cytokines, matrix-degrading enzymes, and chemokines [18]. Inhibition of PBEF could significantly decrease the secretion of TNF-α, IL-1 and IL-6 from inflammatory cells, the inhibitors of PBEF had anti-inflammatory and anti-cancer activity and were finding use as therapeutic agents [19]. It has also been found to play a role in atherosclerosis and to be extensively linked to acute lung injury [20, 21]. It was demonstrated that PBEF participated in ARDS, increased PBEF expression involved epigenetic regulation with hsa-miR-374a and hsa-miR-568, which representing novel therapeutic strategies to reduce inflammatory lung injury [22].

In this study, we established hypoxia/re-oxygenation model and explored the relationship between PBEF and VEGF and p-MLC through RNAi silencing PBEF gene. We found that PBEF siRNA could inhibit the expression of PBEF in HUVEC cells effectively, the expression levels of PBEF, VEGF and p-MLC were significantly increased after Hypoxia/re-oxygenation. When the PBEF gene was knockdown, the expression of VEGF in hypoxia/re-oxygenation was significantly lower, which indicating that PBEF may change the cellular permeability by regulating the expression of VEGF.

Hypoxia/re-oxygenation model simulated ischemia and reperfusion injury in vitro, it was still different from extracorporeal circulation. We will study the effect of PBEF on permeability of pulmonary vascular endothelial cell in vivo in future, we also continue to explore what signal pathway involved in increased permeability caused by PBEF after CPB.

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Disclosure of conflict of interest

None.

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