Functional and metabolic characterization of endothelial cells in chronic thromboembolic pulmonary hypertension

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CHAPTER 7

THE INFLAMMATORY PROFILE OF CTEPH DERIVED ENDOTHELIAL CELLS IS A POSSIBLE DRIVER OF DISEASE PROGRESSION

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ABSTRACT

Chronic thromboembolic pulmonary hypertension (CTEPH) is a form of pulmonary hypertension characterized by the presence of fibrotic intraluminal thrombi causing obliteration of the pulmonary arteries. Although both endothelial cell (EC) dysfunction and inflammation are linked to CTEPH pathogenesis, regulation of the inflammatory response of ECs in CTEPH is not fully understood. Therefore, in the present study we investigate the role of NF-κB proinflammatory signaling pathway in ECs in CTEPH.

To study if NF-κB is activated, pulmonary endarterectomy (PEA) specimens from CTEPH patients and ECs isolated from PEA specimens were stained for phospho-NF-κB-P65. Especially the vessels within the thrombus and CTEPH-ECs are positive for phospho-NF-κB-P65. Moreover, in CTEPH-ECs, IL-8, IL-1β, MCP-1, CCL5, and VCAM-1 mRNA levels were upregulated compared to controls. To assess the involvement of NF-κB signaling in the inflammatory activation, CTEPH-ECs were incubated with the NF-κB inhibitor Bay 11-7085. The increase of pro-inflammatory cytokines was abolished when cells were incubated with the NF-κB inhibitor .

In summary we show that CTEPH-ECs have a pro-inflammatory status and that blocking NF-κB signaling reduced the production of inflammatory factors in CTEPH-ECs. Therefore our results show that the increased pro-inflammatory status of CTEPH-ECs is, at least partially, regulated through activation of NF-κB signaling.

Keywords: Chronic thromboembolic pulmonary hypertension – inflammation – nuclear factor-κB signaling – endothelial dysfunction

INTRODUCTION

Chronic thromboembolic pulmonary hypertension (CTEPH) is a severe cause of pulmonary hypertension (PH) resulting from unresolved pulmonary emboli (PE) that obstruct main pulmonary arteries. In addition, vascular remodeling of muscular pulmonary arteries similar to the arteriopathy observed in pulmonary arterial hypertension (PAH) is found in CTEPH [1-3]. CTEPH patients, without medical intervention, have poor prognosis with a 5-year survival rate between 10-30%, depending on the mean pulmonary artery pressure [4]. Pulmonary endarterectomy (PEA), to remove fibrotic organized clots from pulmonary arteries, is the gold standard treatment for eligible patients with CTEPH and significantly improves patients' survival and hemodynamics [5]. The invasiveness of PEA surgery together with insufficient effects of additional treatment options for inoperable patients and for patients with recurrent/persistent PH (up to 35%) after PEA point out the importance of resolving new, potential curative, targets in CTEPH pathogenesis [4].

Only 75% of patients with CTEPH have history of symptomatic acute PE [6]. To date, the molecular and cellular mechanisms behind the lack of thrombus resolution and vascular remodeling that result in CTEPH remain unclear. The frequently observed remodeling in non-occluded arteries and small pulmonary arteries similar to that observed in PAH supports the presence of endothelial dysfunction in CTEPH pathogenesis [2]. Studies have reported the involvement of endothelial cells (ECs) in the process of thrombi organization and remodeling of surrounding pulmonary arteries through impaired angiogenesis, changes in EC function and increased production of inflammatory cytokines and adhesion molecules [7-12]. The pulmonary endothelium is an important interface between the circulating blood and underlying tissues. Through the production and release of cytokines, chemokines and adhesion molecules it controls inflammatory cell adhesion and trafficking [13]. Disturbances in endothelial inflammatory processes are central in cardiovascular disease development and progression including PH. Studies, both in PAH lungs and animal models of PAH, showed crucial involvement of inflammation in pulmonary vascular remodeling [14-19]. However, the regulation of inflammation in CTEPH-EC is largely unknown.

The expression and production of inflammatory mediators in the endothelium is controlled by nuclear factor (NF)-κB, a central regulator of inflammation [20,21]. Activated NF-κB translocates into the nucleus to promote expression of target genes such as tumor necrosis

factor alpha (TNFα), interleukin-1-beta (IL-1β), IL-8, monocyte chemoattractant protein 1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) among others [20]. Several of these endothelial-derived inflammatory factors have shown to influence cell survival, proliferation and migration of cells within the vascular wall and thereby contributing to vascular remodeling [10,21-23]. Therefore, blocking nuclear translocation of NF-κB using inhibitory small molecules like Bay 11-7085 [24], may be a method to study the involvement of the NF-κB pathway in the regulation of inflammatory pathways in CTEPH-ECs.

Here, we hypothesized that ECs from CTEPH patients exhibit a pro-inflammatory status through sustained activation of the NF-κB pathway which contributes to EC dysfunction and abnormalities involved in CTEPH pathophysiology.

MATEREIAL AND METHODS

Study population and samples collected

This study included CTEPH-ECs derived from 8 different endarterectomy specimens from patients with CTEPH who underwent pulmonary endarterectomy at the Hospital Clinic of Barcelona, Spain. The study was approved by the institutional Ethics Committee of the Hospital Clínic of Barcelona and informed consent was obtained from all patients. All patients were diagnosed according to the 2015 ESC/ERS Guidelines [25].

Pulmonary endothelial cell isolation and culture

ECs isolated from endarterectomy specimens, referred to as CTEPH-ECs, were cultured as previously described [26]. In short, ECs were plated onto 0.1% gelatin-coated wells and grown in endothelium cell medium (ScienceCell Research Laboratories) supplemented with endothelial cell growth supplement, 5% fetal bovine serum (FBS) and Penicillin/Streptomycin solution (ScienceCell Research Laboratories). The cell phenotype was characterized by staining the cells with antibodies against a panel of endothelial and smooth muscle cell-specific markers, including endothelial nitric oxide synthase (eNOS) and alpha smooth muscle Actin (αSMA) [26]. Patients characteristics are presented in supplementary Table 1. Three different batches of human pulmonary artery endothelial cells (HPAEC) (Lonza, CC-2530) were used as control cells.

Gene expression analysis

The levels of IL-8, MCP-1, C-C motif chemokine ligand 5 (CCL5), IL-1β, ICAM-1 and VCAM-1 were measured from CTEPH-EC cultured in low serum conditions (endothelial cell medium with 0.1% FBS) by real-time quantitative PCR (n=8 per group). Bay 11-7085 (Cayman Chemicals, 14795, USA), a potent NF-κB inhibitor, was applied at 1μM final concentration. Treatments were performed in endothelial cell medium with 0.1% FBS and stimuli were provided for 6 hours. Total RNA was extracted using the ReliaPrep™ RNA Cell Miniprep system (Promega) and concentrations were determined by spectrophotometry. Reverse transcription was performed using a RevertAid First strand cDNA synthesis kit (ThermoFisher Scientific). For qRT-PCR, QuantiTect® SYBR® Green PCR kit (Qiagen) and specific primers were used on the ViiA7 Real-Time PCR system (Applied Biosystems). Relative quantification was calculated by normalizing the Ct (threshold cycle) of the gene of interest to the Ct of an endogenous control (TBP and ARP) in the same sample, using the comparative ΔΔCt method. All primers were produced by Invitrogen and primer sequences can be found in supplementary Table 2.

Immunostaining

Paraffin-embedded sections (5μm) were incubated at 4˚C overnight with antibodies against phospho-NF-κB P65 (rabbit anti-phospho-p65, 1:100; Signalway antibody, 11260), platelet endothelial cell adhesion molecule (goat anti-CD31, 1:1000; R&D Systems AF3628). Sections were then incubated with anti-rabbit Alexa Fluor 555 (Invitrogen, A31572) or anti-goat Alexa Fluor 647 (Invitrogen, A21447) secondary antibodies for 2 hours. Nuclei were counterstained using Hoechst 34580 (1:800; Sigma-Aldrich, 63493). Sections were imaged using slidescanner 3DHistech Pannoramic 250.

CTEPH-EC and HPAEC were seeded at 1 x 10^5 cells/mL in 24-well plates pre-coated with 1% gelatin and covered with glass cover slides and allowed to grow for 48 hours. Cells were washed with cold PBS and fixated with 4% paraformaldehyde. Next, cells were permeabilized with PBS/0.25% Triton and blocked with PBS/0.1% Triton/10% FBS. Next, slides were incubated at 4˚C overnight with antibodies against phospho-P65 (rabbit anti-phospho-P65 1:100; Signalway antibody, 11260) and CD31 (mouse anti-CD31 1:250; Dako, M0823). The slides were then incubated with anti-rabbit Alexa Fluor 488 (Invitrogen, A21206) or anti-mouse Alexa Fluor 647 (Invitrogen, A21447) secondary antibodies for 1 hour. Nuclei were

counterstained with DAPI (ProLong™ Gold Antifade Mountant with DAPI, Invitrogen, P36931). Slides were imaged using a Leica DM6B microscope and the mean fluorescence intensity was quantified using ImageJ software.

Statistical analysis

Results are described as mean ± standard deviation and were compared using unpaired t-test (immunofluorescence analysis) or unpaired t-test with Welch's correction (gene expression analysis). Statistical analyses were performed using GraphPad Software. P-values <0.05 were considered statistically significant.

RESULTS

Endothelial localization of phospho-P65 in CTEPH specimen

Pulmonary endarterectomy specimens were stained for the presence of phospho-NF-κB P65 (pP65) and PECAM/CD31. Thrombus vessels present in the tissue stained positive for PECAM/CD31. Interestingly, in these areas pP65 showed to colocalize with PECAM/CD31, indicating the presence of pP65 ECs lining vessels within the thrombus. Cells positive for pP65, but negative for PECAM/CD31, were also observed throughout the tissue. These cells are most likely infiltrating inflammatory cells (**Figure 1A-B**).

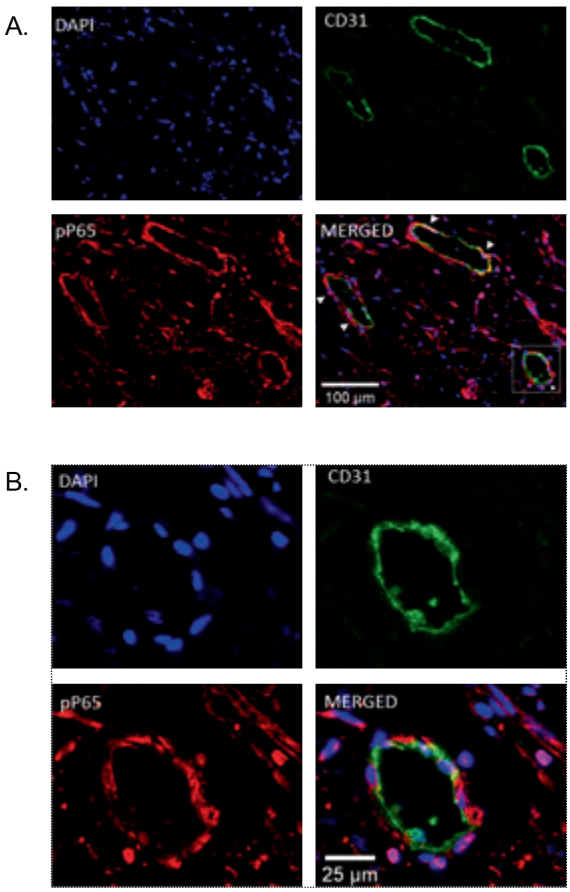

Figure 1. PEA immunofluorescence. (A) Localization of phospho-NF-κB P65 (pP65) in vessels in endarterectomy specimens from patients with CTEPH, using double labeling with PECAM (green) and pP65 (red). **(B)** pP65 immunoreactivity was observed in endothelial cells from vessels within the thrombus (magenta, indicated by the white arrows). Nuclei were counterstained with DAPI (blue). Scale bar 100µm (panel A) and 25µm (panel B).

Fluorescence staining of phospho-P65 in cultured CTEPH-ECs

NF-κB activation in CTEPH-ECs was studied by monitoring nuclear translocation of the p65 subunit by immunofluorescence. Cultured CTEPH-ECs, isolated from four different pulmonary endarterectomy specimens, and three different control HPAEC were stained for PECAM/CD31 and pP65. Both CTEPH-ECs and HPAECs were positive for endothelial marker PECAM/CD31 (**Figure 2A**). The number of cells showing nuclear anti-p65 was measured from random selected areas of the coverslip. CTEPH-ECs and HPAECs showed positive fluorescence signal for pP65 which was mainly found within DAPI positive nuclei (**Figure 2A**). The amount of nuclear translocation of the pP65 subunit was determined by quantifying the intensity of the fluorescence signal inside the nuclei, and CTEPH-ECs showed a trend towards a 2.4 fold higher (p=0.06) nuclear signal intensity compared to control cells (**Figure 2B**).

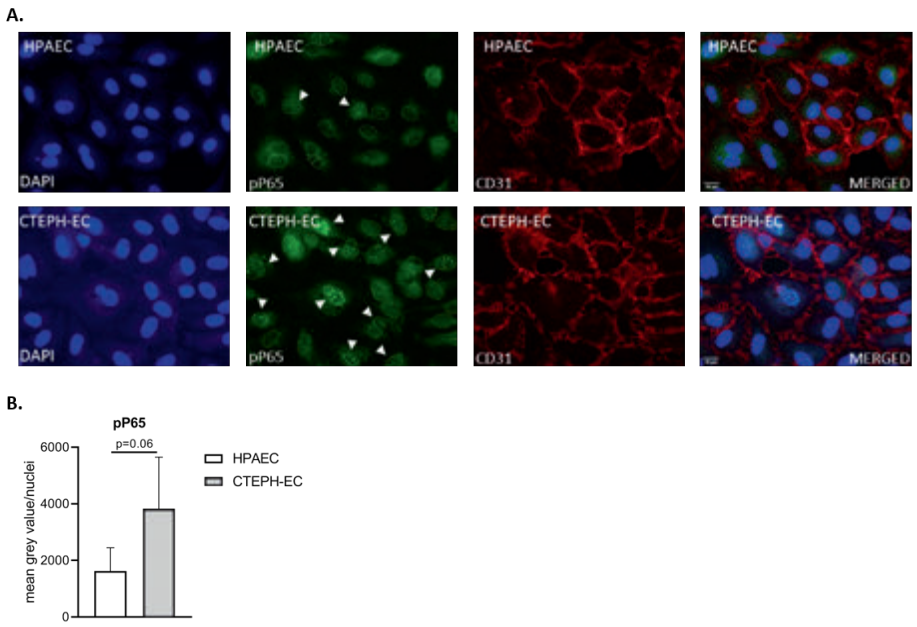

Figure 2. Fluorescence staining of phospho-P65 in cultured ECs. (A) HPAECs (top) and CTEPH-ECs (bottom) were stained for endothelial marker PECAM/CD31 (red) and phospho-NF-κB P65 (pP65; green). Both ECs showed presence of PECAM/CD31 and nuclear pP65 staining (indicated by the white arrows). Nuclei were counterstained with DAPI, scale bar 10μm. **(B)** Presence of nuclear PP65 was quantified in both CTEPH-ECs and HPAECs. CTEPH-ECs showed 2.4 fold higher presence of nuclear PP65 compared to control cells (p=0.06) (unpaired t-test); CTEPH-ECs, n=4; HPAECs, n=3 Data is expressed as mean ± SD.

Expression of pro-inflammatory cytokines in CTEPH-ECs

To investigate the role of increased NF-κB activation in CTEPH-ECs, cytokines downstream the NF-κB signaling pathway were measured. mRNA expression levels of IL-8 and MCP-1 showed a 5.5 fold (p=0.009) and a 2.5 fold (p=0.05) increase, respectively, compared to control cells. CCL5 showed a 7 fold increase (p=0.03) in mRNA expression levels compared to control cells. ICAM-1 showed a trend towards increased levels in CTEPH-ECs but did not reach significance (p=0.07). IL-1β showed a 6 fold increase (p=0.02) in mRNA levels compared to HPAECs. At last, mRNA levels of VCAM-1 showed a 3 fold (p=0.03) increase in CTEPH-ECs compared to control cells (**Figure 3**).

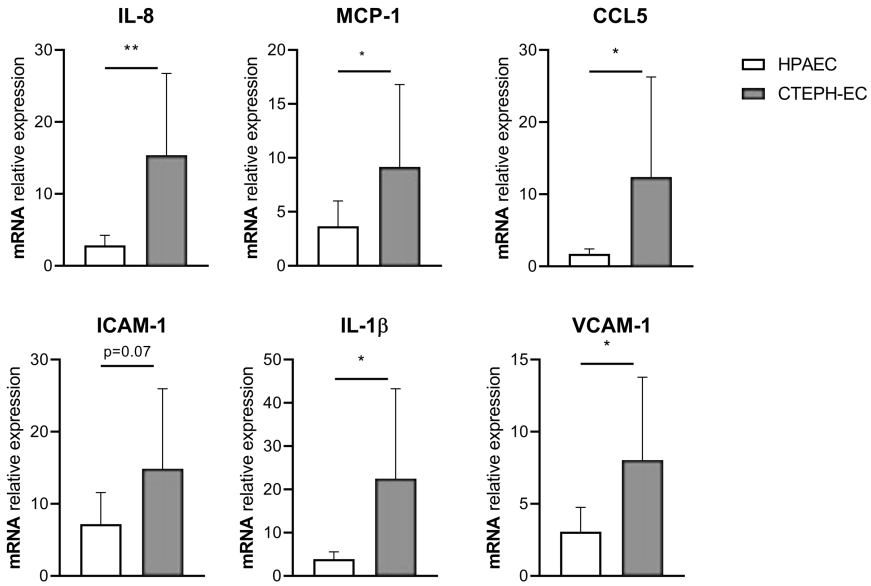

Figure 3. Inflammatory cytokines in cultured CTEPH-ECs. mRNA expression levels of IL-8, IL-1β, MCP-1, CCL5, VCAM-1 and ICAM-1 were found increased in CTEPH-ECs compared to HPAECs. CTEPH-EC, n=8; HPAEC, n=3; unpaired t-test with Welch's correction , p<0.05 = *, data is expressed as mean ± SD.

Effect of NF-κB inhibition in CTEPH-ECs

To examine if a reduction in NF-κB activity leads to reduced expression of inflammatory cytokines, cultured CTEPH-ECs were incubated with 1μM Bay 11-7085, a NF-κB inhibitor, which at the same time demonstrates the role of NF-κB signaling on the expression of pro-inflammatory cytokines. VCAM-1 showed a 1.6 fold reduction (p=0.02) in mRNA levels in CTEPH-ECs compared to control condition without inhibitor. ICAM-1 and MCP-1 showed a tendency towards reduction after incubation with Bay 11-7085 but did not reach significance (p=0.09 and p=0.08, respectively). mRNA levels of IL-8 were found not changed in CTEPH-ECs after incubation with Bay 11-7085 compared to the control condition (**Figure 4**).

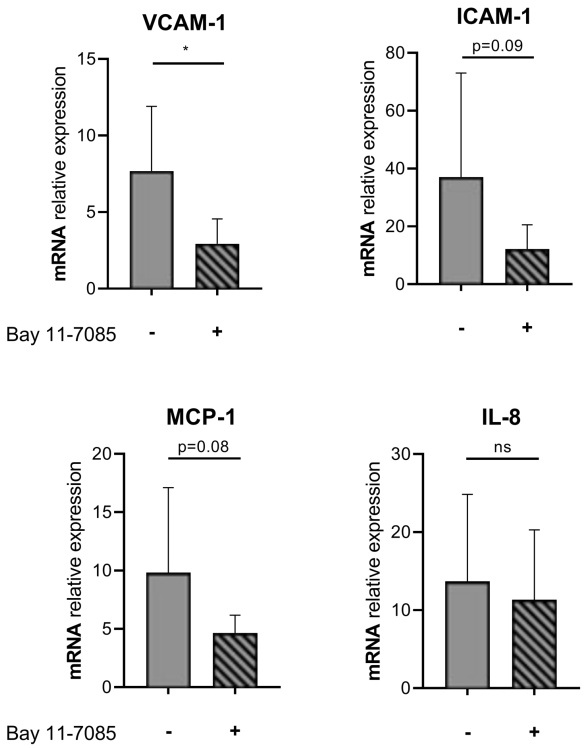

Figure 4. Effect of NF-κB inhibition on CTEPH-ECs. Cultured CTEPH-ECs were incubated with NF-κB inhibitor Bay 11-7085 at a final concentration of 1μM. VCAM-1 showed a significant reduction in mRNA levels compared to control condition without Bay. mRNA levels of ICAM-1 and MCP-1 showed a trend towards reduction after Bay 11-7085 incubation. IL-8 mRNA expression levels were found similar between CTEPH-ECs incubated with Bay 11-7085 and without. CTEPH-ECs, n=5; unpaired t-test, $p<0.05$ = *, data is expressed as mean ± SD.

DISCUSSION

In this study we showed the presence of pP65 positive thrombus vessels in CTEPH PEA specimens. Furthermore, we showed increased nuclear localization of pP65 in cultured CTEPH-ECs indicating the presence of more active NF-κB signaling in CTEPH-EC compared to control HPAECs. Increased expression of IL-8, IL-1β, CCL5, MCP-1 and VCAM-1, all inflammatory factors downstream of NF-κB signaling pathway, in CTEPH-ECs compared to controls confirmed this observation. Furthermore, CTEPH-ECs incubated with the NF-κB inhibitor Bay 11-7085 showed a decrease in the expression of VCAM-1, ICAM-1 and MCP-1.

Our observation that cytokines are elevated in CTEPH-ECs is in line with a comprehensive analysis by Zabini *et al.* where they found that the expression of inflammatory cytokines, including IL-6, IL-8 and MCP-1, was significantly higher in serum and PEA tissue of CTEPH patients compared to healthy controls and healthy lung tissue, respectively [27]. Despite the clear evidence of an inflammatory component in CTEPH, the regulation of the inflammatory response of ECs in CTEPH is less studied and only few studies have been performed to better understand the regulatory pathways of inflammation in CTEPH pathogenesis. Ataam *et al*. recently reported that increased ICAM-1 contributes to EC dysfunction in CTEPH [11]. In the present study we show for the first time that cultured CTEPH-ECs have increased nuclear phospho-p65 compared to control cells. NF-κB activation involves besides phosphorylation and degradation of inhibitory proteins IκBs, also phosphorylation of the P65 subunit. This phosphorylation is an important event to enhance the transcriptional capacity of DNA bound NF-κB and activates the transcription of key targets VCAM-1, ICAM-1 and MCP-1 in ECs [28-30]. Therefore, our results indicate that the observed increase in phosphorylation of the P65 subunit is key in the elevated expression of inflammatory cytokines in CTEPH-ECs. Several inflammatory-related diseases such as cancer, atherosclerosis, restenosis and asthma have been associated with increased activation of NF-κB and expression of its downstream mediators [31-35]. Based on the increase in NF-κB activation observed, we hypothesized that inhibition of NF-κB signaling could reverse the pro-inflammatory profile in CTEPH-ECs. In the current study we found that inhibition of NF-κB signaling, by blocking the phosphorylation of NF-κB inhibitor IκB-α with inhibitory small molecule Bay 11-7085, results in reduced expression of inflammatory cytokines VCAM-1, ICAM-1 and MCP-1 in CTEPH-ECs. Cancers such as multiple myeloma, where NF-κB signaling plays a significant role in the pathogenesis,

have been successfully treated with drugs that have NF-κB as their primary or secondary target [36]. These findings confirm that the increased inflammatory cytokines in CTEPH-ECs are, at least partially, regulated through NF-κB signaling.

Based on the results obtained in this study we can conclude that CTEPH-ECs have a pro-inflammatory status as shown by the increased production of inflammatory cytokines IL-8, MCP-1, IL-1β, CCL5, ICAM-1 and VCAM-1. More importantly, we showed that the increased inflammatory cytokines observed in CTEPH-ECs are, at least partially, regulated through NF-κB signaling and that blocking NF-κB activation might be an important target in CTEPH to prevent disease progression or recurrent PH.

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Supplementary Table 1. Clinical features and hemodynamic parameters

	CTEPH (n=8)
Female/male	5/3
Age years	63.15 ± 10.88
BMI kg·m^{-2}	25.97 ± 4.35
mPAP mmHg	42.13 ± 9.52
PVR dyn·s·m^{-5}	706.75 ± 230.07
PAOP mmHg	10.25 ± 3.77
Cardiac index L·min^{-1}·m^{-2}	2.23 ± 0.61
Right atrial pressure mmHg	9.38 ± 4.63
SvO2 %	59.50 ± 7.86
6MWD m	398.13 ± 102.02
BNP pg·mL^{-1}	209.54 ± 360.12
WHO FC	
I	0
II	2
III	6

Data are presented as n or mean ± SD. CTEPH: chronic thromboembolic pulmonary hypertension; BMI: body mass index; mPAP: mean pulmonary artery pressure; PVR: pulmonary vascular resistance; PAOP: pulmonary artery occlusion pressure; SvO2: mixed venous oxygen blood saturation; 6MWD: 6-minute walking distance; BNP: brain natriuretic peptide; WHO FC: world health organization functional class

Supplementary Table 2. Primer sequences

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
IL8	CTGGCCGTGGCTCTCTTG	CTTGGCAAAACTGCACCTTCA
MCP1	CTGTGCCTGCTGCTCATAG	AGCTTCTTTGGGACACTTGC
CCL5	GCATCTGCCTCCCCATATTC	AGTGGGCGGGCAATGTAG
IL-1β	CGAATCTCCGACCACCACTAC	TCCATGGCCACAACAACTGA
ICAM	CTGCAGACAGTGACCATC	GTCCAGTTTCCCGGACAA
VCAM	CAGGCTGGAAGAAGCAGA	GGCCTTTCGGATGGTATAGG
ARP	CACCATTGAAATCCTGAGTGATGT	TGACCAGCCGAAAGGAGAAG
TBP	TGGAAAAGTTGTATTAACAGGTGCT	GCAAGGGTACATGAGAGCCA