

Original Article

Inhibition of ER stress by 4-PBA protects MCT-induced pulmonary arterial hypertension via reducing NLRP3 inflammasome activation

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Abstract: NLRP3 inflammasome activation and endoplasmic-reticulum (ER) stress contribute to the pathogenesis and development of pulmonary arterial hypertension (PAH). Recently studies have shown that ER stress induced NLRP3 inflammasome activation in chronic liver diseases. But the mechanism is still unclear, especially the role of ER stress and relationship with NLRP3 inflammasome in monocrotaline MCT-induced PAH. To identify the cellular mechanism of ER stress mediate NLRP3 inflammasome during MCT-induced PAH, we investigated the influence of classic ER stress inhibitor 4-phenyl butyric acid (4-PBA) on ER stress and NLRP3 inflammasome, which partially affect the activation of inflammation, PAH was induced by injecting MCT for 4 weeks. The rats were randomly assigned to control group, MCT group, and 4-PBA treatment group. We demonstrated that inhibition of ER stress by 4-PBA, which further prevented the activation of the NLRP3 inflammasome, decreased the release of the pro-inflammatory mediators IL-1 β , IL-6 and TNF- α . Moreover, it was found that 4-PBA decreased LPS-induced PAECs injury through decreasing activation of NLRP3 inflammasomes and expression of inflammatory factors. Taken together, our study indicated that inhibition of ER stress by 4-PBA protects the MCT-induced PAH through decreasing activation of NLRP3 inflammasomes and release of pro-inflammatory mediators.

Keywords: Pulmonary arterial hypertension, NLRP3, inflammasome, endoplasmic reticulum stress, 4-PBA, pulmonary artery endothelial cell

Introduction

Pulmonary arterial hypertension (PAH) is a deadly disease in which vasoconstriction and vascular remodeling both lead to a progressive increase in pulmonary vascular resistance, right ventricular failure [1]. Very little is known about the determining molecular and cellular mechanism in the pathophysiology of PAH. Some researches indicate that abnormal apoptosis and proliferation of vascular endothelium and smooth muscle cells are involved in the remodeling process of the pulmonary arteries [2, 3]. Structural changes in the pulmonary vasculature include dysfunction of pulmonary artery endothelial cells (PAECs) and increased wall thickness of pulmonary arterioles [4, 5]. Furthermore, the function of the endothelium is

altered in PAH, resulting in an imbalance between endothelium-derived vasoconstrictors and proliferative agents [6], in addition to contributing to the remodeling process. Therefore, PAECs have been suggested to play an important role in pulmonary hypertension and vascular remodeling. But the abnormal dysfunction and mechanism of PAECs are still under controversy.

PAH is now seen as a vasculopathy in which structural changes driven by excessive vascular inflammation, along with the recruitment and infiltration of circulating cells. But, the role and importance of inflammation and immune activation in the development of pulmonary hypertension are not fully understood. Inflammasomes are large, cytosolic multi-protein complex

consisting of an inflammasome sensor molecule [such as the nucleotide-binding oligomerization domain like receptor (NLR)], caspase-1, and often an adaptor protein called apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) [7, 8]. The NLRP3 inflammasome is the most fully characterized inflammasome [9]. Upon activation, inflammasome assembly triggers the activation of caspase-1, which in turn cleaves pro-IL-1 β , leading to the release of activated IL-1 β , which are important mediators of innate immune responses. Some studies showed that increased circulating levels of IL-1 β and IL-18 have been observed in patients with pulmonary arterial hypertension, indicating that inflammasome can be activated in this condition [8, 10]. Another study showed that ASC-deficient mice exposed to hypoxia, develop less increase in right ventricle (RV) systolic pressure (RVSP) and reduced RV remodeling compared with wild-type (WT) controls [11], indicating that NLRP3 inflammasome is important for development of pulmonary hypertension.

The endoplasmic reticulum (ER) is an important subcellular organelle in eukaryotic cells, which has essential roles in multiple cellular processes that are required for cell survival and normal cellular functions [12]. Prolonged perturbation of ER leads to ER stress and unfolded protein response (UPR) and contributes to the pathogenesis of various chronic disorders including pulmonary arterial hypertension [13, 14]. Recent studies have reported that reduction of endoplasmic reticulum stress by 4-PBA prevents the development of hypoxia-induced pulmonary arterial hypertension [15]. In livers from obese mice, administration of LPS or tunicamycin results in IRE1 α and PERK activation, leading to the overexpression of CHOP, activates the NLRP3 inflammasome, subsequently initiating hepatocyte pyroptosis (caspase-1, -11, interleukin-1 β secretion) and apoptosis [16]. In HK-2 cells Ang II-induced increases in the expression of NLRP3, ASC, caspase-1 and IL-1 β , were significantly inhibited by pretreatment with the ER stress inhibitor 4-PBA [17]. However, the role of ER stress and its relationship with inflammasome in PAH are largely unknown to date.

In this study, the classic ER stress inhibitor 4-PBA was added to suppress the ER stress signaling pathways in MCT-induced PAH rat

models. In addition, we also employed 4-PBA and ER stress inducer thapsigargin (TG) to further evaluate the effect of ER stress on NLRP3 inflammasome in LPS-stimulated cultured pulmonary artery endothelial cells. Our data demonstrated that 4-PBA might protect MCT-induced pulmonary arterial hypertension through inhibiting ER stress-triggered NLRP3 inflammasome activation both *in vivo* and *in vitro*.

Materials and methods

Animal model and tissue preparation

Male Wistar rats (220-250 g) were obtained from the Animal Center of the Chinese Academy of Science (Shanghai, China). Rats were randomized into 3 groups consisting of control group (n=10), MCT group (n=10), 4-PBA (Sigma Aldrich, USA) +MCT group (n=10). PAH was induced by intraperitoneally administering MCT (Sigma-Aldrich; 60 mg/kg) once, on the first day of the experiment, as previously described [18]. Rats in the control group received the same volume of saline. In the 4-PBA+MCT group, PAH rats received 4-PBA starting on the day of PAH induction and continuing for four weeks. The 4-PBA was administered daily by gavage at a dose of 500 mg/kg body weight in the 4-PBA+MCT groups. The lung tissues were flash frozen in liquid nitrogen and stored at -80°C until they were used for real-time PCR and Western blot analysis.

Hemodynamics

After four weeks, animals were anesthetized with pentobarbital (60 mg/kg intraperitoneally) before they were subjected to the hemodynamic study, pulmonary arterial pressures and right ventricular pressure were measured using the BL-420F biological and functional information collection system (TME, Chengdu, China).

Pulmonary vascular remodeling

After completion of hemodynamic measurements, then sacrificed by exsanguination for histological examination [19]. The excised lung and heart were divided. Next, the right ventricular free wall (RV) and the left ventricular plus septal (LV+S) wall were separated and weighed. The right ventricular hypertrophy index (RVHI) was calculated as [RV/(LV+S)], yielding a right ventricular remodeling parameter in PAH.

Lung tissues were fixed in formalin for histology studies. After being embedded in paraffin, samples were sliced and stained with hematoxylin and eosin (H&E). For each lung sample, the pathological morphology and the pulmonary arteriole of each group were randomly selected and observed under a light microscope.

RNA extraction and real-time PCR quantitation

The mRNA levels were assessed by Real-Time PCR. In brief, the total RNA was extracted with Trizol according to manufacturer's protocol (Invitrogen, Carlsbad, CA) and reverse-transcribed using Gen Amp RNA PCR kit (Applied Biosystems, Foster City, CA). Quantitative Real-Time PCR was performed on an ABI 7500 Real-Time PCR thermocycler, whereas SYBR green PCR Master Mix (Applied Biosystems) was used for Real-Time PCR analysis. The relative quantities of target transcripts were calculated from duplicate samples after normalization of the data against the housekeeping gene, β -actin. Dissociation curve analysis was performed after PCR amplification to confirm the specificity of the primers. Relative mRNA expression was calculated using the Ct method. The following primer pairs were used: the specific primers: NLRP3: upstream 5'-CCCCGTGAGTCCCA-TTA-3', downstream 5'-GACGCCAGTCCAAC-AT-3'; ASC: upstream 5'-GAAGCTGCTGACAGTG-CAAC-3', downstream 5'-GCCACAGCTCCAGACT-CTTC-3'; β -actin: upstream 5'-GGCCAACCGT-GAAAAGATGA-3', downstream 5'-GACCAGAGG-CATACAGGGACAA-3'.

Enzyme-linked immunosorbent assay (ELISA)

The cytokine production of IL-1 β , IL-6 and TNF- α in serum was quantified using a murine ELISA development kit (PEPROTECH, Rocky Hill, NJ) according to the manufacturer's instruction.

Cell culture

Pulmonary arterial endothelial cells (PAECs) were isolated from digested whole lung tissue using CD31 Ab-coated magnetic beads (Dyna-beads; Invitrogen). Lung tissues were obtained from 3 months healthy Wistar rat. Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 IU/mL penicillin, 100 μ g/mL streptomycin at 37°C in a 5% CO₂/95% air environment. PAECs phenotype

was confirmed by positive immunostaining for EC markers CD31. Cells from passages 4-6 were used for each of the experiments. Cells were incubated for at least 24 hrs in serum-free, before each of the experiments.

Cell proliferation assay

The viability of the PAECs was determined by MTT assays. Briefly, cells were placed in a 96-well plate at a density of 1×10^4 cells/well in 200 μ l of complete medium for 24 hours or treated with LPS at different dose (0, 1, 5, 10, 20 μ g/ml). Each treatment was repeated in five wells. MTT reagent (20 μ l, 5 mg/ml in PBS; Sigma) was added to each well and incubated for 4 h. Then MTT solution was removed from the wells by aspiration, and the formazan crystals were dissolved in dimethylsulfoxide (150 μ l). Absorbance was recorded at 570 nm wavelength.

To determine the effect of ER stress on PAECs apoptosis treated by LPS, PAECs were seeded on 6-well plates (2×10^5 cells/well) and the classic ER stress inhibitor 4-phenyl butyric acid (4-PBA, 5 mM) and ER stress inducer thapsigargin (TG, 10 μ M) was added in cultured PAECs for 1 hours followed by treatment with LPS for 24 hours. Cells were washed and harvested; apoptotic rates were measured using a PI/Annexin V-FITC kit (Invitrogen) and analyzed by FACSscan flow cytometer (Becton Dickinson).

Immunoblotting

Proteins were extracted from the lung tissues samples and PAECs cells using Radio Immunoprecipitation Assay (RIPA) buffer (Beyotime, P0013B) with PMSF lysis buffer. Proteins were separated on 10%-12% SDS-PAGE gels and were then transferred to PVDF membranes (Immobilon, IPVH00010). Membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS) for 1.5 hr and probed with antibodies ATF6 (Abcam), GRP78 (Cell Signal), CHOP (Cell Signal), NLRP3 (Cell Signal), ASC (Enzo Life Sciences), anti-pro-caspase-1 (Cell Signal), anti-cleaved-caspase-1 (Cell Signal), anti-pro-IL-1 β (Santa Cruz Biotechnology), anti-cleaved-IL-1 β (Cell Signal) and β -actin (Abcam) overnight at 4°C. After three washes, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Beyotime Institute of Biotechnology) for 1 hr at

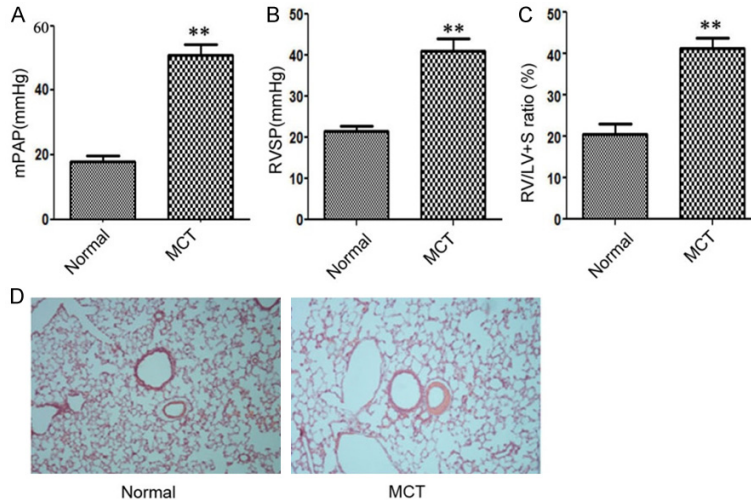


Figure 1. MCT induced pulmonary arterial pressure and pulmonary vascular remodeling. (A) mPAP (mmHg) and (B) RVSP (mmHg) were observed in rats in the Normal and MCT groups. (C) MCT-induced RV hypertrophy indicated as RV/(LV+S) ratio. Compared to the Normal group, significant changes in mPAP, RVSP and RV/(LV+S) ratio were observed in MCT group. Data represent means \pm SD ($n=7$). $**P < 0.01$ versus Normal group. (D) Lung sections of the rats were stained with representative H&E for histological examination (magnification $\times 20$). MCT significantly increased pulmonary arterial wall thickness; muscularization and luminal stenosis were observed.

room temperature. The relative intensities of bands were analyzed by using image analysis software program (Image J 1.44p, Wayne Rasband, National Institutes of Health, USA).

Statistical analysis

All data were expressed as the mean \pm SEM of three independent experiments and each experiment included triplicate sets. Student's *t* test was used for statistical analysis for comparison of two Groups. For more than two groups, statistical evaluation of the data was performed using a one-way analysis-of-variance (ANOVA) test, followed by Tukey's post hoc test. Statistical significance was accepted at $P < 0.05$.

Results

MCT triggers NLRP3 inflammasome activation and endoplasmic reticulum stress in rats

Inflammation has emerged as an important player in MCT-induced PAH. Firstly, the model of MCT-induced PAH rat was evaluated hemodynamics and H&E staining. Catheterization was performed successfully in survival animals. After 4 weeks in MCT injection conditions, rats

developed a moderate PAH. Rats ($n=7$) in the MCT group had higher mPAP values (47.28 ± 3.45 mmHg) than rats in the control group (18.99 ± 2.63 mmHg) (Figure 1A). Meanwhile, rats treated with MCT had higher RVSP values than rats in the control group (41.06 ± 2.15 mmHg and 23.79 ± 2.84 mmHg for the MCT and control groups, respectively) (Figure 1B). Additionally, we observed right ventricular hypertrophy (RVH) measured as the weight ratio of right ventricle/left ventricle plus septum (RV/LV+S). The ratio between the RV and LV+S weight was of $21.17 \pm 1.60\%$ in control rats. Thickness of the RV wall in the MCT group was significantly increased compared with the control value ($40.73 \pm 3.23\%$, $P < 0.01$), as shown in Figure

1C. Lung histology in the PAH group showed increased medial wall thickness of pulmonary arteries, muscularization and luminal stenosis when compared with that of control rats (Figure 1D), that is consistent with the changes in hemodynamic parameters.

Because the NLRP3 inflammasome is one of the best understood members of the inflammasome family, we next tested the expression of NLRP3 and the adaptor protein apoptosis associated speck like protein (ASC) after treatment with MCT. As shown in Figure 2A-D, based on the RT-PCR and Western blot analysis of lung tissue, the mRNA and protein expression levels of NLRP3 and ASC were markedly increased after MCT treatment for 4 weeks ($P < 0.05$).

Previous studies demonstrated a close relationship between the NLRP3 inflammasome and endoplasmic reticulum stress. Therefore, we hypothesized that ER stress may be involved in MCT-induced NLRP3 inflammasome activation. We examined upstream targets GRP78, downstream ATF6 and CHOP in the ER stress signaling pathway. We found that expression of protein ATF6, GRP78 and CHOP, were upregulated in the MCT treated group, compared with control group (Figure 2E and 2F). These data

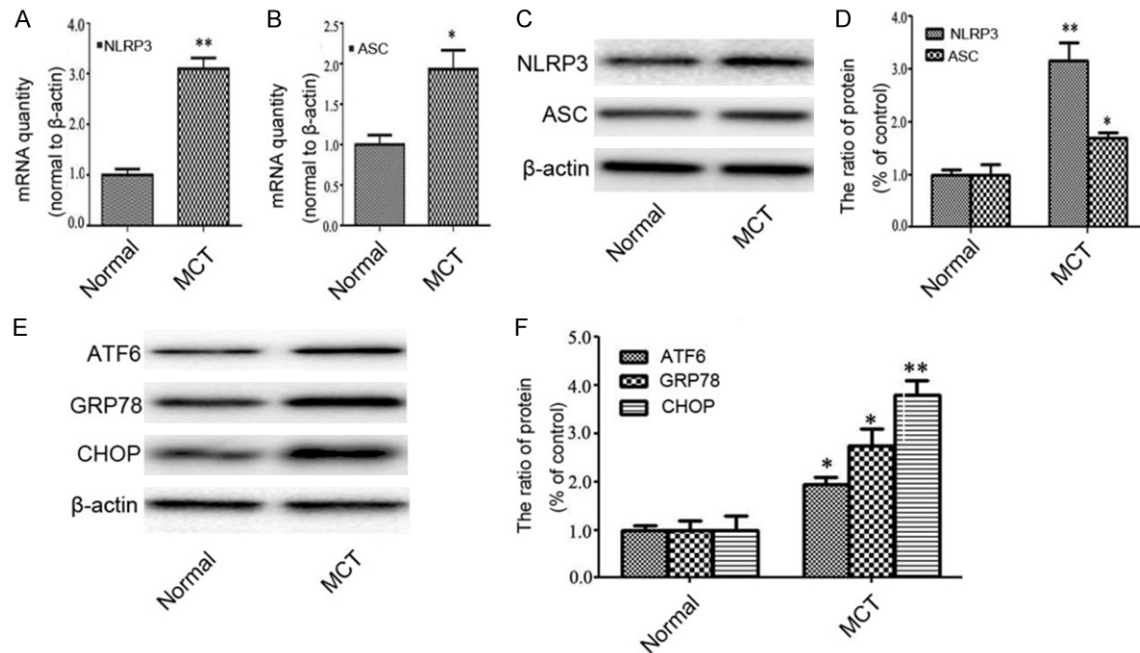


Figure 2. MCT triggers NLRP3 inflammasome activation. (A, B) Real-time PCR and Western blotting (C, D) analysis show that the mRNA quantity and protein expression of NLRP3 and ASC were markedly increased after treatment with MCT compared with the Normal group. (E) The ER stress relative proteins ATF6, GRP78 and CHOP were analyzed by Western blotting. (F) The bar graph indicates the relative abundance of the protein (normalized to that of β -actin). Bars represent mean \pm SE (n=7). * P < 0.05, ** P < 0.01 versus Normal group.

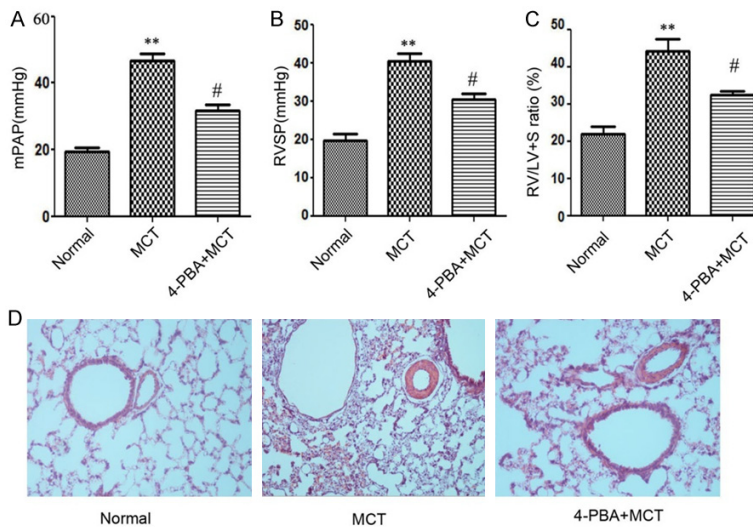


Figure 3. 4-PBA decreases MCT-induced pulmonary arterial pressure and pulmonary vascular remodeling. (A) mPAP (mmHg) and (B) RVSP (mmHg) were observed in the Normal, MCT, and 4-PBA+MCT groups. (C) MCT-induced RV hypertrophy indicated as RV/(LV+S) ratio. Data represent means \pm SD (n=7). # P < 0.05 versus MCT group. (D) Lung sections of the rats were stained with representative H&E for histological examination (magnification \times 20). 4-PBA significantly decreased pulmonary arterial wall thickness, muscularization and luminal stenosis induced by MCT.

pulmonary vascular remodeling.

4-PBA decreases MCT-induced pulmonary arterial pressure and pulmonary vascular remodeling

To determine whether 4-PBA could protect against MCT-induced PAH, the mPAP, RVSP values and ratio of RV/LV+S were detected. We found that MCT-increased the mPAP was decreased to 34.37 \pm 2.45 mmHg after 4 weeks of the 4-PBA administration, compared with MCT group (47.15 \pm 2.38 mmHg) (**Figure 3A**). 4-PBA group showed lower RVSP than MCT group (44.32 \pm 3.35 mmHg vs. 32.16 \pm 3.98 mmHg) (**Figure 3B**). Meanwhile, RV/LV+S ratio in the 4-PBA rats was significantly decreased, compared with

MCT rats (32.26 \pm 1.14% vs. 40.73 \pm 3.75%, P < 0.05) (**Figure 3C**). Lung histology showed that

demonstrated that ER stress and inflammasome activation were involved in MCT-induced

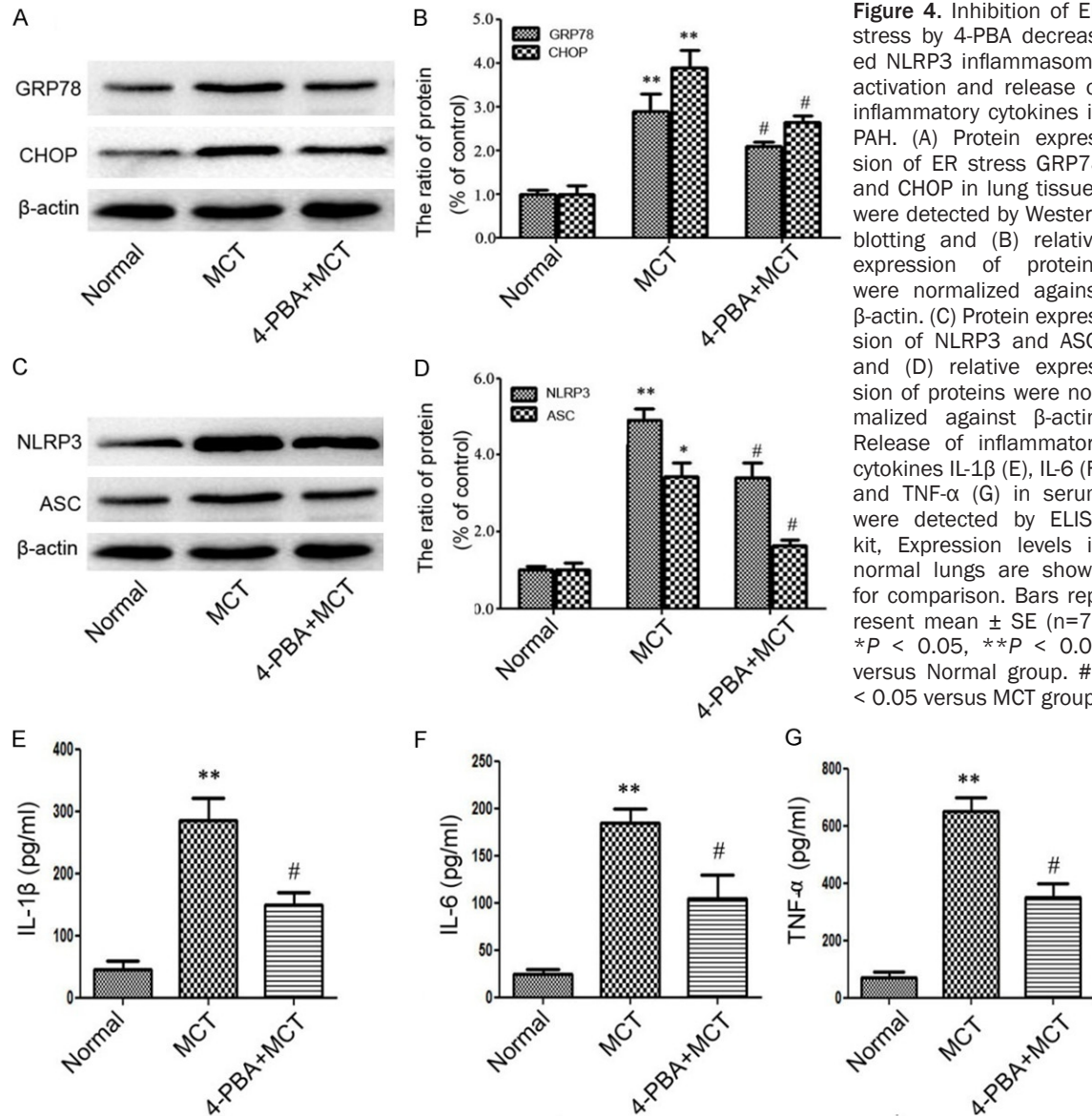


Figure 4. Inhibition of ER stress by 4-PBA decreased NLRP3 inflammasome activation and release of inflammatory cytokines in PAH. (A) Protein expression of ER stress GRP78 and CHOP in lung tissues were detected by Western blotting and (B) relative expression of proteins were normalized against β-actin. (C) Protein expression of NLRP3 and ASC, and (D) relative expression of proteins were normalized against β-actin. Release of inflammatory cytokines IL-1β (E), IL-6 (F) and TNF-α (G) in serum were detected by ELISA kit. Expression levels in normal lungs are shown for comparison. Bars represent mean ± SE (n=7). **P* < 0.05, ***P* < 0.01 versus Normal group. #*P* < 0.05 versus MCT group.

MCT-increased medial wall thickness of pulmonary arteries, muscularization and luminal stenosis, which was clearly attenuated with 4-PBA treatment (**Figure 3D**). These data demonstrated that 4-PBA protects MCT-induced pulmonary vascular remodeling.

4-PBA mediated reduction of ER stress decreased NLRP3 inflammasome activation in PAH

To evaluate whether ER stress plays a vital role in regulating NLRP3 inflammasome activation, we used 4-PBA, a chemical chaperone that reduces ER stress. First, the protein expression levels of GRP78 and CHOP were determined to

confirm the effect of 4-PBA. Compared to MCT group, we observed that the protein levels of GRP78 and CHOP induced by MCT, whereas it was significantly decreased with 4-PBA, as shown in **Figure 4A** and **4B**. Next, whether NLRP3 inflammasome activation in MCT rats is related to increased ER stress. Western blot results showed that inhibition of ER stress by 4-PBA obviously inhibited MCT-induced expression of NLRP3 and ASC (**Figure 4C** and **4D**). Meanwhile, release of inflammatory cytokines IL-1β (151.2 ± 30.6 pg/ml vs. 285.5 ± 52.8 pg/ml, *P* < 0.05), IL-6 (104.6 ± 43.5 pg/ml vs. 185.2 ± 24.7 pg/ml, *P* < 0.05) and TNF-α (351.5 ± 65.4 pg/ml vs. 650.2 ± 59.3 pg/ml, *P*

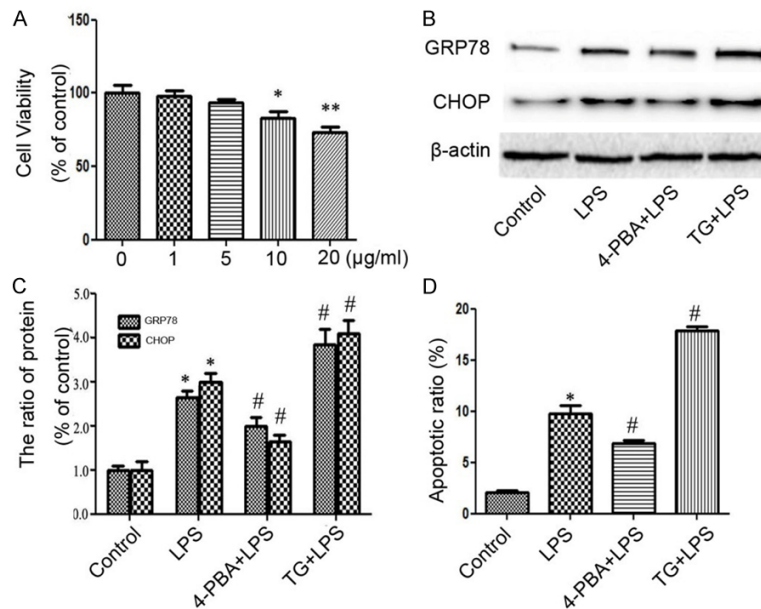


Figure 5. Effect of ER stress on the cell viability in cultured pulmonary artery endothelial cells. PAECs were pretreated with or without 3-MA (5 mM) or TG (10 µM) for 1 hours, then treated with LPS for 24 hours. (A) Cell viability was determined by MTT assay. (B) ER stress related proteins GRP78 and CHOP were analyzed by Western blotting. (C) The bar graph indicates the relative abundance of each protein (normalized to that of β-actin). (D) The effect of 4-PBA and TG on LPS-induced cell apoptosis. Cells were stained with PI and Annexin V-FITC, and the positive stained cells were counted using FACSscan. Representative results of three independent experiments. * $P < 0.05$ versus control group, # $P < 0.05$ versus LPS group.

< 0.05) in the serum of MCT-treated rats also markedly decreased 4-PBA (Figure 4E-G), which suggests that ER stress plays an important role in regulating inflammasome activation and release of inflammatory cytokines.

Effect of ER stress on the cell viability in cultured pulmonary artery endothelial cells

Injury of pulmonary arterial endothelial cells is key pathological process of MCT-induced PAH. In order to mimic *in vivo* model, LPS was used in cultured PAECs. PAECs were incubated for 24 hours at dose (1, 5, 10, 20 µg/ml) of LPS. The effect of LPS on the viability of PAECs was determined by MTT assay. Treatment of the cells with LPS at concentration of 10 and 20 µg/ml resulted in significant decrease in cell viability, whereas treatment of the cells with LPS at 1 and 5 µg/ml did not significantly affect cell viability ($P > 0.05$) (Figure 5A). Based on this result, we chose 10 µg/ml LPS as the concentration used in further experiment.

To further confirm that the role of ER stress in injury of PAECs induced by LPS, the classic ER

stress inhibitor 4-PBA and ER stress inducer TG were added in cultured PAECs. The expression of ER stress markers, GRP78 and CHOP were detected by Western blot analysis. As shown in Figure 5B and 5C, LPS significantly increased the expression of GRP78 and CHOP proteins in PAECs, and pre-treatment with 4-PBA markedly inhibited the up-regulation of the GRP78 and CHOP proteins induced by LPS ($P < 0.05$). However, treatment with TG further increased protein levels of GRP78 and CHOP induced by LPS.

Furthermore, cells were subjected to PI and Annexin V-FITC staining, the apoptotic cell population was detected by flow cytometry analysis as shown in Figure 5D. The results showed that LPS increased cell apoptosis to 9.9% ($P < 0.05$). Treatment with LPS combined with 4-PBA blocked

LPS-induced classic apoptosis to 5.8% ($P < 0.05$). In contrast, TG resulted in an increase in PAECs, apoptotic cells accounted for 17.4% of the total population ($P < 0.05$). These results indicated that activation of ER stress pathways is key process in LPS-induced PAECs apoptosis.

Regulation of ER stress on NLRP3 inflammasome activation in cultured pulmonary artery endothelial cells

To confirm the effect of ER stress on NLRP3 inflammasome activation induced by LPS in PAECs, we treated PAECs with LPS in the presence or absence of 4-PBA or TG. The expression of protein NLRP3 and ASC between the four groups were confirmed by Western blot (Figure 6A and 6B). As expected, in LPS group, the protein expression of NLRP3, ASC were dramatically upregulated. Activated caspase-1 contributes to maturation of IL-1β [20-22]. Western blot results showed that LPS also increased ratio of active caspase-1/β-actin, increased expression of IL-1β. Treatment with

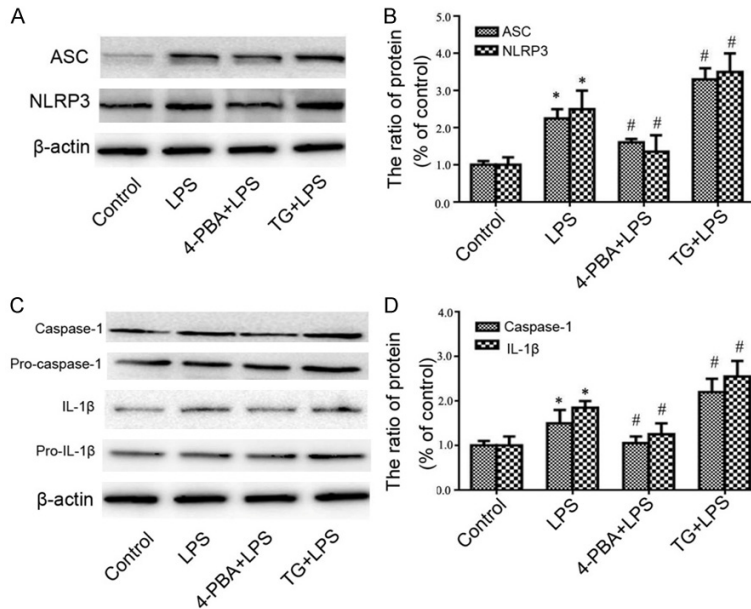


Figure 6. Effect of ER stress on NLRP3 inflammasome activation in cultured pulmonary artery endothelial cells. PAECs were pretreated with or without 3-MA (5 mM) or TG (10 μ M) for 1 hours, then treated with LPS for 24 hours. (A) The proteins expression of ASC and NLRP3 were observed in PAECs. (B) Relative expression of protein was normalized against β -actin. (C) The proteins expression of Caspase-1 and IL-1 β were observed in PAECs. (D) Relative expression of proteins was normalized against β -actin. Compared to the LPS group, administration of 4-PBA significant lowered the proteins expression, however, TG increased protein levels of ASC, NLRP3, Caspase-1 and IL-1 β induced by LPS. Representative results of three independent experiments. * P < 0.05 versus control group, # P < 0.05 versus LPS group.

4-PBA significantly decreased expression of protein NLRP3, ASC, active caspase-1 and IL-1 β . On the contrary, ER stress inducer TG obviously exacerbated LPS-induced NLRP3 inflammasome activation in PAECs (Figure 6C and 6D), which suggests that ER stress plays a crucial role in regulating inflammasome activation.

Discussion

Pulmonary arterial hypertension (PAH) is a refractory syndrome that causes restricted flow through the pulmonary arterial circulation. It has been characterized by inflammatory of the endothelium of small pulmonary arteries and development of pulmonary artery remodeling, associated with thickening and hyperplasia of the intimal and medial layers. The complete pathogenesis of the MCT-induced disease leading to PAH in the Wistar rat remains unknown. In the present study, administration of MCT in rats can cause pulmonary arterial remodeling

in rats with PAH. The data from our study indicate that mPAP, RVSP and RV/(LV+S) ratio were increased by MCT; MCT-treated rats developed right heart failure with increased RV/(LV+S) ratio and medial hypertrophy relative to the control group. These observations are consistent with previous studies [23].

Inflammation plays a key role in human PAH as well as in experimental models. In response to injury and stress, injury of pulmonary arterial endothelial cells produce inflammatory mediators, thereby stimulate the release of cytokines, lead to vascular smooth muscle cells proliferation and migration, vascular remodeling by matrix remodeling, collagen deposition in PAH [24, 25], finally lead to increased pulmonary resistance and right heart failure. The NLRP3 inflammasome is activated in several types of lung injury. Some studies

shown that NLRP3 was significantly up-regulated in the lung of MCT-treated rats compared with the control group [26]. Wu et al. suggested that the alveolar macrophage NLRP3 inflammasome may sense lung alveolar stretching to induce the release of IL-1 β [27]. NLRP3-deficient mice display a suppressed inflammatory response and blunted lung epithelial cell apoptosis in response to hyperoxia induced acute lung injury [28]. In present study, we found that MCT increased the protein levels of NLRP3 and adaptor protein ASC, further induced the release of pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α . Our findings are in accordance with previous studies implying that NLRP3 inflammasome was involved in the process of MCT-induced PAH.

ER stress is an important cellular defense and is evolutionarily conserved. However, persistent or strong ER stress can also trigger apoptosis. Previous studies shown that ER stress markers GRP78 and GRP94 protein levels were

significantly increased in the MCT-induced PAH or hypoxia-induced PAH model [15, 29]. In present study, MCT increased ER stress marker protein expression of GRP78 and GRP94 in rats. Meanwhile, protein levels of ATF6 were increased by MCT treatment. Our results are consistent with previous studies. These results showed that MCT-induced ER stress is related to activation of ATF6 branch.

The potential interplay between ER stress and inflammasome engagement has yet to be explored in different diseases [16, 30]. Recent studies shown that high glucose-induced ER stress increased NLRP3 inflammasome activation in adipose tissue and differentiated 3T3 adipocytes, the main known role of the inflammasome is to produce active caspase-1, which in turn converts pro-IL-18 and pro-IL-1 β into their mature forms [31]. Hypoxia-induced pulmonary hypertension in WT mice is characterized by increased levels of active caspase-1, mature IL-18, and mature IL-1 β in the lung, suggesting that inflammasome activation is a feature of the pathology in PAH [11]. In present studies, we found that NLRP3 inflammasome and ER stress were all activated by MCT treatment. Consistent with previous findings, inhibition of ER stress by 4-PBA decreased mPAP and RVSP values induced by MCT, and alleviated pulmonary arterial remodeling in rats with PAH. Furthermore, we found that 4-PBA inhibited ER stress as well as attenuated protein levels of NLRP3 and ASC in MCT-induced PAH rats, then prevented release of pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α in rats. The pulmonary endothelial cell is considered to be the main target for the MCT intoxication [32]. Meanwhile, MCT-induced disease is a PAH inflammatory model. Therefore, LPS as inflammatory inducer was used in inducing PAECs injury. We found that results *in vivo* were further demonstrated in LPS-induced pulmonary arterial endothelial cells injury. LPS-induced ER stress and activation of NLRP3 inflammasome contributed to PAECs apoptosis. Inhibition of ER stress by 4-PBA attenuate LPS-induced increased protein levels of NLRP3 and ASC. The main known role of the inflammasome is to produce active caspase-1, convert pro-IL-1 β into their mature forms. We showed that LPS increased levels of active caspase-1, mature IL-1 β in PAECs, whereas 4-PBA strongly prevented activation, then protected injury of

PAECs induced by LPS. Induction of ER stress by TG aggravated PAECs apoptosis induced by LPS through increasing protein levels of NLRP3 and ASC, active caspase-1, mature IL-1 β . These results further confirm our theory that Inhibition of ER stress by 4-PBA protects the MCT-induced PAH through reducing activation of NLRP3 inflammasomes and release of inflammatory factors.

In conclusion, our results showed that ER stress leads to NLRP3 inflammasome activation, thus resulting in severe PAH, and contributing as a novel mechanism of ER stress-mediated lung injury. Blocking ER stress-dependent NLRP3 inflammasome by 4-PBA may attenuate the expression and release of inflammatory factors, which protect pulmonary vascular wall damage in PAH.

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Disclosure of conflict of interest

None.

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