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H_2S inhibits pulmonary arterial endothelial cell inflammation in rats with monocrotaline-induced pulmonary hypertension

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This study aimed to determine whether hydrogen sulfide (H_2S) inhibits pulmonary arterial endothelial inflammation in rats with monocrotaline (MCT)-induced pulmonary hypertension and its possible mechanisms. Twenty-four male Wistar rats were divided randomly into control, MCT, and MCT+H_2S treatment groups. Human pulmonary arterial endothelial cells (HPAEC) were cultured and divided into four groups: control, MCT, MCT+H_2S, and H_2S. Pulmonary artery pressure was determined using a right cardiac catheterization procedure 3 weeks after MCT administration. Pulmonary vascular morphological changes and inflammatory infiltration were measured. Endogenous H_2S levels, cystathionine-γ-lyase (CSE) expression, and inflammatory cytokines were determined both *in vivo* and *in vitro*. In addition, phosphorylation of NF-κB p65 and IκBα was detected by western blotting, and NF-κB p65 nuclear translocation, as well as its DNA-binding activity, was determined. Pulmonary hypertension and vascular remolding developed 3 wks after MCT administration, with elevated lung tissue inflammatory infiltration and cytokine level associated with activation of the NF-κB pathway, both *in vivo* and *in vitro*. However, the endogenous H_2S/CSE pathway was downregulated in MCT rats. By contrast, an H_2S donor markedly reduced pulmonary artery pressure, pulmonary vascular structural remolding, and increased lung inflammatory infiltration and cytokine levels of MCT-treated rats. Meanwhile, H_2S reversed the activation of the NF-κB pathway successfully. The downregulated pulmonary arterial endothelial H_2S/CSE pathway is involved in the pulmonary inflammatory response in MCT-treated pulmonary hypertensive rats. H_2S attenuated endothelial inflammation by inhibiting the NF-κB pathway.

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Pulmonary arterial hypertension (PAH), a common clinical disorder complicated by a variety of cardiovascular pulmonary diseases, presents as increased pulmonary artery pressure and pulmonary vascular remodeling, which often has a serious impact on the development of primary disease and prognosis. However, its pathogenesis remains unclear.[1]

Several recent studies have reported the essential role of pulmonary vascular inflammation in PAH.[2] When subjected to various stimulants, including hypoxia, shear stress, inflammation, or toxins, pulmonary vascular endothelial cells release large amounts of vasoactive molecules and inflammatory mediators, initiating the pulmonary vascular inflammation cascade, which promotes abnormal pulmonary vascular contraction and vascular remodeling, leading to pulmonary hypertension.[3] Therefore, further clarification of the mechanism underlying pulmonary vascular inflammation is essential for a complete understanding of the pathogenesis of PAH and would aid the development of novel therapeutic strategies.

Pulmonary vascular endothelial cell injury has been established as a key event in the initiation of pulmonary vascular inflammation. Many studies of molecular genetics, pathophysiology, and neurohumoral regulation in the development of pulmonary inflammation have been conducted. Severe damage to the vascular endothelium, and neo-intimal and inflammatory infiltration of pulmonary vasculatures, have been observed in patients with pulmonary hypertension.[4] Indeed, animal studies demonstrated that anti-inflammatory treatment could protect against PAH.[5] However, the regulatory mechanisms governing the synthesis

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and release of inflammatory cytokines in pulmonary arterial endothelial cells, as well as the subsequent pulmonary vascular inflammation in the development of PAH, have yet to be fully revealed.

Hydrogen sulfide (H_2S), previously known as an air pollutant, is produced endogenously by cystathionine-γ-lyase (CSE), cystathionine-β-synthase and 3-mercaptopyruvate sulfurtransferase in mammals. In the cardiovascular system, H_2S is generated primarily by CSE and has an important role in the maintenance of cardiovascular homeostasis via dilating vessels, inhibiting vascular smooth muscle cell proliferation, promoting cardiomyocyte apoptosis and preventing collagen remodeling. The abnormal generation and dysfunction of H_2S is a crucial event in the development of several important cardiovascular diseases, such as primary hypertension, pulmonary hypertension, atherosclerosis, vascular calcification, and cardiac hypertrophy. To date, endogenous H_2S has been identified as the third gasotransmitter to be involved in cardiovascular regulation, following nitric oxide and carbon monoxide.[6,7] Our previous studies showed that H_2S attenuated pulmonary hypertension and pulmonary vascular remodeling by inhibiting pulmonary artery smooth muscle cell proliferation, promoting apoptosis of pulmonary artery smooth muscle cells, and reducing collagen deposition.[8] Tao *et al.*[9] found that H_2S promoted vascular endothelial cell proliferation and angiogenesis, and Wang *et al.*[10] discovered that H_2S inhibited vascular endothelial cell inflammation in the context of atherosclerosis. However, whether and how H_2S has a regulatory role in pulmonary vascular endothelial inflammation remain unclear.

The NF-κB pathway is the canonical event in the development of the inflammatory response. In the resting state, NF-κB dimers (p65/p50) bind with the inhibitory protein IκBα in the cytoplasm. When subjected to inflammatory insult, phosphorylation of IκBα is initiated, which is then decomposed through the ubiquitin pathway, releasing NF-κB p65; the free NF-κB p65 then combines with specific inflammation-related gene to regulate their transcription.[11] NF-κB target genes regulate a broad family of inflammatory cytokines, for example adhesion molecules, chemokines, cytokines, tumor necrosis factor (TNF-α), intercellular adhesion molecule-1 (ICAM-l), interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1).[12] Notably, H_2S was reported to inactivate the NF-κB signaling pathway in ox-LDL-stimulated monocyte-macrophage cells.[13] Therefore, we aimed to determine whether H_2S could inhibit the NF-κB signaling pathway, thereby inhibiting inflammatory gene expression and antagonizing pulmonary vascular inflammation in PAH.

Monocrotaline (MCT) is a two-pyrrole alkaloid that is transformed by P450 monooxygenase in the liver into its active form, MCT pyrrole (MCTP). Circulating MCTP acts selectively on pulmonary arterial endothelial cells, leading to irreversible cell injury, vascular inflammation, and remodeling, which has led to the use of MCT to produce a pulmonary hypertension model.[14] Thus, the present study was designed to develop a PAH rat model using MCT and a pulmonary artery endothelial cell model using MCTP. Using *in vivo* and *in vitro* experiments, we investigated the changes in the endogenous H_2S/CSE pathway and then examined the impact of H_2S on pulmonary arterial endothelial cellular inflammation and vascular remodeling. The NF-κB pathway has been identified as vital branch point; therefore, its associated transduction pathways were explored to reveal the pathophysiological effect of H_2S on pulmonary vascular inflammation and remodeling.

MATERIALS AND METHODS

Establishment of Rats with MCT-Induced PH

The present study was approved by Peking University First Hospital Animal Ethics Committee (J201326) and all experimental operations conformed to the animal ethics guidelines. Twenty-four 6-week-old healthy male Wistar rats were divided randomly into the control group, MCT group, and MCT+H_2S group ($n=8$ for each group) after adaptive breeding. On the first day, MCT rats and MCT+H_2S rats were administered with MCT (60 mg/kg) by intraperitoneal injection, while the control rats were injected with same dose of saline.[15] The MCT+H_2S rats were injected daily with the H_2S donor, NaHS (56 μmol/kg) for 21 days, while the control rats and MCT rats were given same dose of saline.

Cell Culture

Human pulmonary arterial endothelial cells (HPAECs) were purchased from *Wuhan Pricell* (WuHah, China); specialized endothelial cell medium was used to culture HPAECs under conventional culture procedures. The fourth to the sixth generation of cells were used for the experiments and were divided randomly into the control group, MCTP group, and MCTP+H_2S group. MCTP cells were treated with 60 μg/ml MCTP, while the controls were treated with the same concentration of dimethyl formamide.[16] MCTP+H_2S cells were preconditioned with 200 μmol/l NaHS for 20 min before incubation with MCTP.

Measurement of Pulmonary Artery Pressure and Right Ventricular Hypertrophy

Rats were anesthetized via intraperitoneal injection of 0.5% sodium pentobarbital (0.1 ml/100 g) after 21 days of drug intervention. The right external jugular vein was exposed and a catheter was guided through the superior vena cava, right atrium, and right ventricle (RV) into the pulmonary artery. The extracorporal end of the catheter was connected to a pressure sensor to record the continuous changes of pulmonary artery pressure, including systolic pulmonary artery pressure, diastolic pulmonary artery pressure, and mean pulmonary artery pressure.[17] Meanwhile, the heart rate of each rat was recorded.

The rats were killed and the rat RV and left ventricle plus septum (LV+SP) were isolated and subsequently flushed with

sterile saline to remove residual blood. Rat heart tissues were weighed using an analytical balance and the RV/(LV+SP) ratio was calculated.

Assessment of Pulmonary Vascular Morphology

The thoracic cavity of rats was exposed after pulmonary artery pressure measurement. The right upper lung lobe was harvested. Paraffin-embedded lung tissue slices of 5 mm in thickness were prepared for hematoxylin and eosin (HE) staining. A light microscope was used to examine the morphological changes, such as vascular remodeling and inflammatory infiltration, in rat lung tissues. The relative medial thickness (RMT) and relative medial areas (RMA) were measured and calculated using a Leica Q550CW imaging system.

Rat lung tissue of 1×1 mm in size was prepared immediately after being killed and fixed in 3% glutaraldehyde for electron microscopy examination. The morphological changes in the pulmonary endothelial cells were analyzed using a JEM-1230 transmission electron microscope.

Detection of H_2S Content *In Vivo* and *In Vitro*

Rat plasma was collected after centrifugation at 13 000 *g* for 20 min. Rat lung tissue was rinsed with cool saline, homogenized, and centrifuged to recover the supernatant. The lung tissue H_2S concentration was calibrated by the protein concentration. The H_2S contents in rat plasma and lung tissue were determined using a TBR4100 radical analyzer, which detects free H_2S.[18]

Culture supernatants were collected for H_2S content measurement after incubation with drugs for 6 h, while HPAECs were fixed in moderate amount of paraformaldehyde for 20 min, rinsed with PBS, and then incubated with H_2S probes (provided by Professor Xinjing Tang, Peking University Health Science Centre, Beijing, China) for 30 min at 37 °C in a dark room. The probes work based on the reduction of azido groups by H_2S, which produces green fluorescence. A fluorescence microscope was used to determine the H_2S level in HPAECs and green fluorescence indicated endogenous H_2S generation. Fluorescence intensity and H_2S content were positively correlated.

ELISA Determination of Inflammatory Cytokine Levels

Inflammatory cytokines, including TNF-α, IL-8, IL-6, ICAM--1, and MCP-1, were measured using enzyme-linked immunosorbent assay (ELISA) kits (Rapidbio, USA). Rat plasma was centrifuged and the supernatant was collected. Rat lung tissue was homogenized in physiological saline at a volume ratio of 1:10 and the protein concentration was determined. Samples and standard substances were incubated separately with an equal volume of diluent in an enzyme-labeled plate at room temperature for 1 h using a shaker. Subsequently, the supernatant was removed, and the cells were rinsed with washing solution and dried. Secondary antibodies were then added and the cells were incubated for 1 h. After rinsing with washing solution, 100 μl of horseradish peroxidase was added to each well to develop the chromogenic reaction. Absorbance at 450 nm as the vertical axis and standard substance concentration as the horizontal axis were used to produce a standard curve. The inflammatory cytokine concentrations in the rat plasma and lung tissue homogenate were then calculated. The concentrations in the homogenate were calibrated using the protein concentration.

Western Blotting

Rat lung tissue homogenate was prepared and then boiled in sample buffer for 5 min to denature the proteins. Protein concentration was then measured using a Bradford kit. Equal amounts of protein (60 μg) were loaded on 10–15% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The primary antibody dilutions were 1:200 for CSE (Cell Signaling Technology, USA), 1:1000 for NF-κB-p65 and p-NF-κB-p65 (Cell Signaling Technology), 1:200 for IκBα and p - IκBα (Santa Cruz Biotechnology, USA), and 1:5000 for β-actin (Control; Kang Cheng, Shanghai, China). Secondary antibodies (Cell Signaling Technology) were used at a dilution of 1:6000. The AlphaImager graphical analysis software (Alpha Innotech Corporation, USA) was used to scan and quantify the blot. NF-κB p65 phosphorylation was reflected by the p-NFκB-p65/NFκB p65 ratio.

Cells were harvested after drug treatment, and the cell lysate was prepared and denatured for western blotting. Protein quantities of ICAM-1 and CSE were detected following the above protocol.

In addition, nuclear and cytoplasmic extracts were prepared using the Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, USA). Western blotting analysis of NF-κB p65 protein expression was conducted as described above and the nuclear translocation of NF-κB p65 was analyzed.

Localization of ICAM-1 and NF-κB in HPAECs by Immunofluorescence

Immunofluorescent imaging was performed under a confocal laser-scanning microscope (TCS SP5, Leica Microsystems, Germany). Briefly, HPAECs were rinsed with PBS before fixation with 4% paraformaldehyde. The HPAECs were then incubated with the anti-p65 NF-κB antibody (1:50, Cell Signaling Technology) and an anti-ICAM-1 antibody (1:50, Santa Cruz Biotechnology) overnight. HPAECs were subsequently incubated with secondary antibody (anti-rabbit-FITC conjugated, Life Technologies Corporation, USA) at 37 °C for 1 h. Coverslips were mounted onto slides using ProLong antifade mounting reagent (Life Technologies Corporation).

EMSA and AM-ELISA Determination of the DNA-Binding Activity of NF-κB

After 1 h of drug intervention, HPAECs were harvested and nuclear extract was prepared using the Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). An electrophoretic mobility shift assay (EMSA) was carried out using a digoxigenin gel shift kit (Roche Applied Science, Germany) to determine DNA-binding activity of NF-κB. Briefly, 10 μg of nuclear protein was subjected to gel electrophoresis and an oligonucleotide with the NF-κB consensus binding sequence 5'-AGTTGAGGGACTTTCCCAGGC-3' was used.[19]

After incubation with drugs for 1 h, the nuclear extract was prepared as described above. Equal amounts of nuclear proteins were analyzed for NF-κB p65 DNA activity using an AM-ELISA kit (Active Motif, USA).

Data Analysis

Data are expressed as means ± s.e. The data of different groups were calculated using one-way ANOVA within the SPSS 19.0 statistical analysis software. $P<0.05$ was considered significant.

RESULTS

The Endogenous H_2S/CSE Pathway was Downregulated in Rats with MCT-Induced Pulmonary Hypertension

Compared with control rats, the H_2S content in the plasma and lung tissue of MCT rats was decreased markedly (both $P<0.01$); however, the H_2S content in the plasma and lung tissue of the MCT+ H_2S rats was increased ($P<0.01$) compared with the MCT rats (Figures 1a and b).

Compared with control rats, CSE expression in MCT rats was downregulated significantly (both $P<0.05$), whereas compared with MCT rats, the CSE expression in MCT+H_2S rats was upregulated significantly (both $P<0.01$; Figure 1c).

H_2S Attenuated Pulmonary Artery Pressure and Vascular Structural Remodeling in Rats with MCT-Induced Pulmonary Hypertension

Compared with the control group, the pulmonary arterial systolic pressure and mean diastolic pressure were increased significantly in the MCT group ($P<0.01$), whereas compared with the MCT group the MCT+H_2S rat pulmonary artery systolic, diastolic, and mean pressures decreased ($P<0.01$; Figures 2a–c). The heart rates among the control group (413.2 ± 55.1 bpm), MCT group (412.0 ± 49.2 bpm), and MCT+H_2S group (424.6 ± 36.6 bpm) were not significantly different ($F=0.171$, $P=0.844$).

Compared with the control group, the RV/(LV+SP) ratio of the MCT group was increased ($P<0.01$), whereas the RV/(LV+SP) ratio in rats in the MCT+H_2S group was decreased compared with the MCT group ($P<0.01$; Figure 2d).

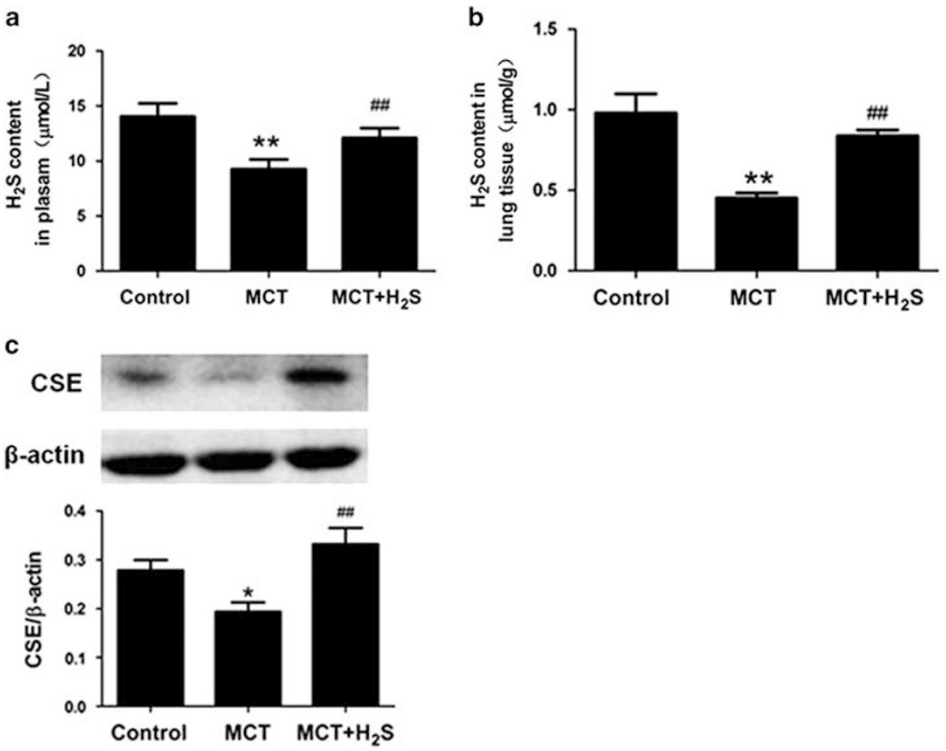

Figure 1 The endogenous H_2S pathway was downregulated in rats with MCT-induced pulmonary hypertension. (**a** and **b**) H_2S content and H_2S content in rat plasma (**a**), H_2S content in rat lung tissue (**b**); (**c**) CSE expression in rat lung tissue. Note: mean ± s.e., $n=8$ in each group, $^{**}P<0.01$, $^{*}P<0.05$ *vs.* Control group, $^{\#\#}P<0.01$ *vs.* MCT group. CSE, cystathionine-γ-lyase; H_2S, hydrogen sulfide; MCT, monocrotaline.

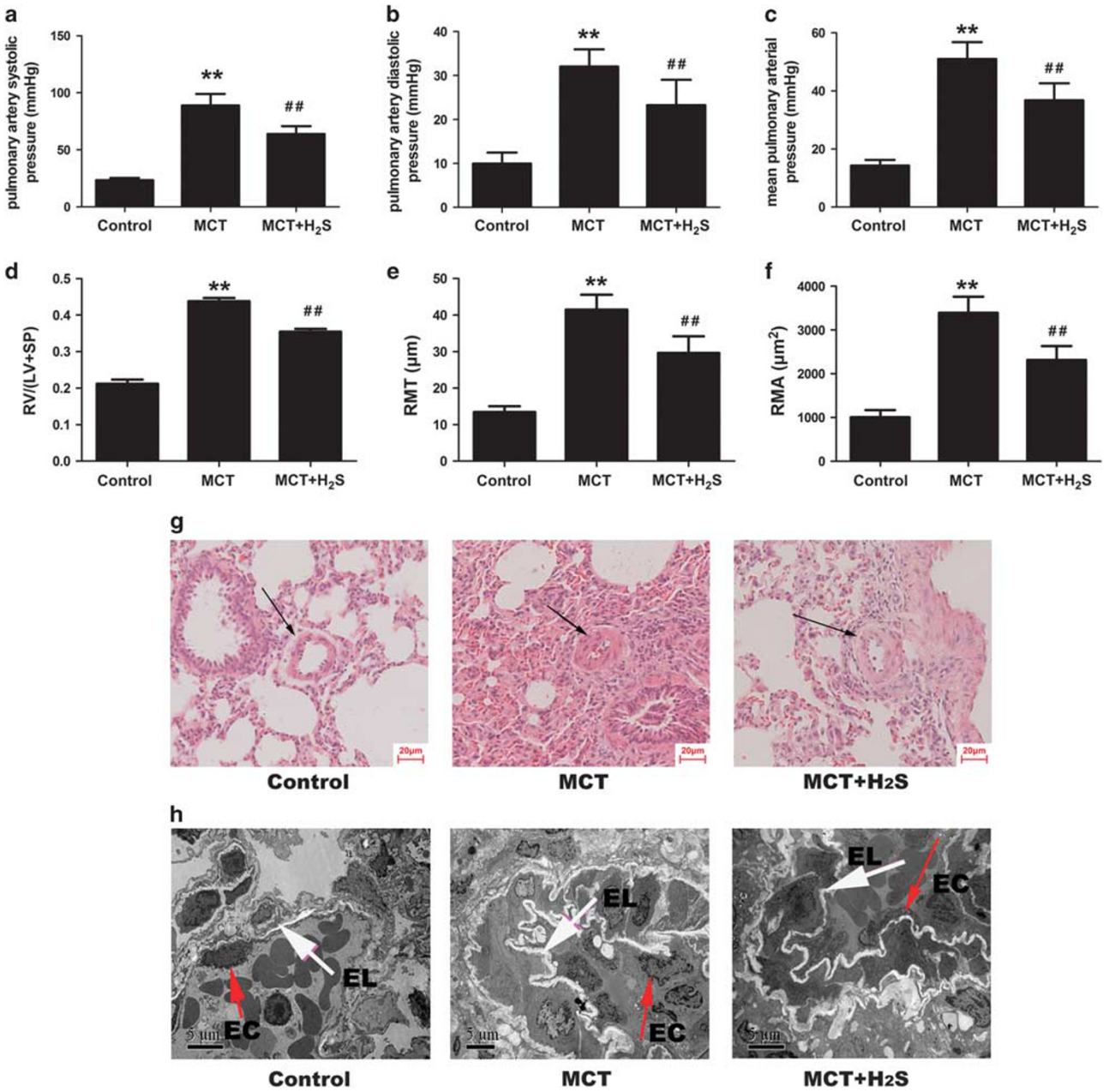

Figure 2 H_2S attenuated pulmonary artery pressure and vascular remodeling in rats with MCT-induced pulmonary hypertension. (**a**) Pulmonary artery systolic pressure of rats in different groups; (**b**) pulmonary arterial diastolic pressure; (**c**) pulmonary arterial mean pressure; (**d**) rat RV/(LV+SP) ratio; (**e**) the RMT of pulmonary vasculatures; (**f**) the RMA of pulmonary vasculatures; (**g**) HE staining of rat lung tissue ×400; H: electron microscope result of rat lung tissue. Red arrows indicate pulmonary arterial endothelial cell, white arrows indicate the pulmonary artery elastic layer. Note: mean ± s.e., $n = 8$ in each group, $^{**}P < 0.01$ *vs.* Control group, $^{\#\#}P < 0.01$ *vs.* MCT group. HE, hematoxylin and eosin; MCT, monocrotaline; RMA, relative medial area; RMT, relative medial thickness.

Microscopic observation of pulmonary structural changes showed that the control rat pulmonary arterial vessel wall was thin, the lumen was clear and there was no inflammatory cell infiltration. The pulmonary artery wall of the MCT group was thick, had a narrowed lumen, and showed significant inflammatory cell infiltration. In the MCT+H_2S group, the pulmonary vascular wall was thin, the lumen was clear, and there was reduced inflammatory infiltration (Figure 2g).

Compared with the control group, the RMT and RMA of the pulmonary artery in the MCT group were increased significantly ($P < 0.01$), whereas the RMT and RMA in the MCT+H_2S group were lower ($P < 0.01$) compared with the MCT group (Figures 2e and f).

Transmission electron microscopy showed that control rats presented intact pulmonary arterial endothelial cells with a complete, continuous internal elastic layer. However, in the

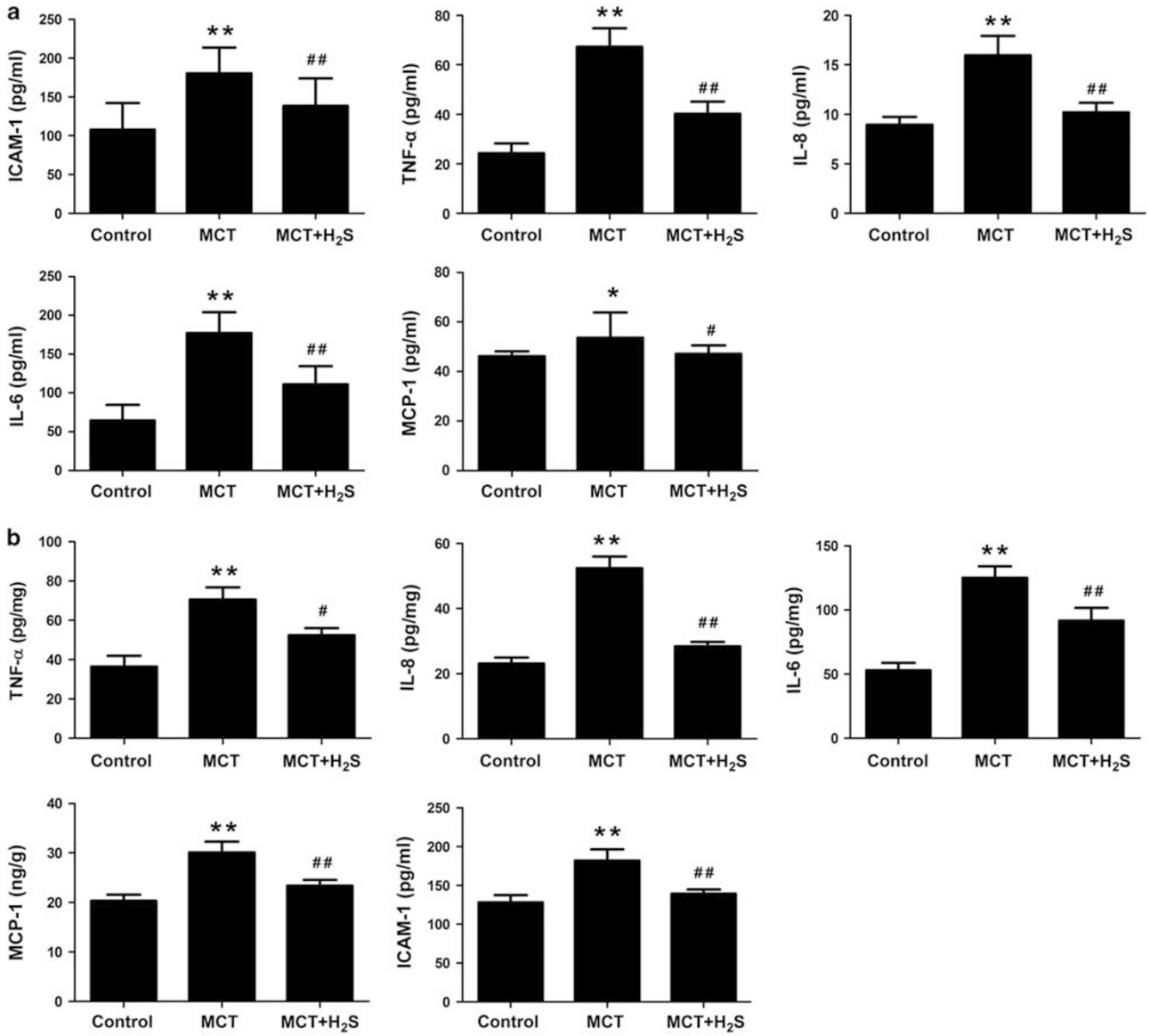

Figure 3 H_2S inhibited vascular inflammation in rats with MCT-induced pulmonary hypertension. (**a**) The levels of ICAM-1, TNF-*α*, IL-8, IL-6, and MCP-1 in rat plasma determined by ELISA. (**b**) The levels of ICAM-1, TNF-*α*, IL-8, IL-6, and MCP-1 in rat lung tissue determined by ELISA. Note: mean ± s.e., $n=8$ for each group, $^{**}P<0.01$, $^{*}P<0.05$ *vs.* Control group, $^{\#\#}P<0.01$, $^{\#}P<0.05$ *vs.* MCT group. ELISA, enzyme-linked immunosorbent assay; H_2S, hydrogen sulfide; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MCT, monocrotaline; TNF, tumor necrosis factor.

MCT group, the pulmonary artery endothelium was swollen, with a widened sub-endothelial space and an irregular internal elastic layer. In the MCT+H_2S group, however, pulmonary arterial endothelial cells and the internal elastic layer were intact (Figure 2h).

H_2S Inhibited Vascular Inflammation in Rats with MCT-Induced Pulmonary Hypertension

The ELISA results showed that, compared with control rats, the levels of ICAM-l, TNF-α, IL-8, IL-6, and MCP-1 in the MCT rat plasma were increased significantly ($P<0.01$, $P<0.01$, $P<0.01$, $P<0.01$, and $P<0.05$, respectively). Meanwhile, the levels of ICAM-l, TNF-α, IL-8, IL-6, and MCP-1 in the plasma of the MCT+H_2S group were decreased significantly compared with the MCT group ($P<0.01$, $P<0.01$, $P<0.01$, $P<0.01$, and $P<0.05$, respectively; Figure 3a).

The levels of ICAM-l, TNF-α, IL-8, IL-6, and MCP-1 in the lung tissue of the MCT group were increased ($P<0.01$); however, in the presence of H_2S, they were markedly decreased ($P<0.01$, $P<0.05$, $P<0.01$, $P<0.01$, and $P<0.01$, respectively; Figure 3b).

H_2S Inhibited NF-κB Activation in Rats with MCT-Induced Pulmonary Hypertension

Compared with the control rats, IκBα phosphorylation was upregulated markedly ($P<0.01$), total IκBα was downregulated

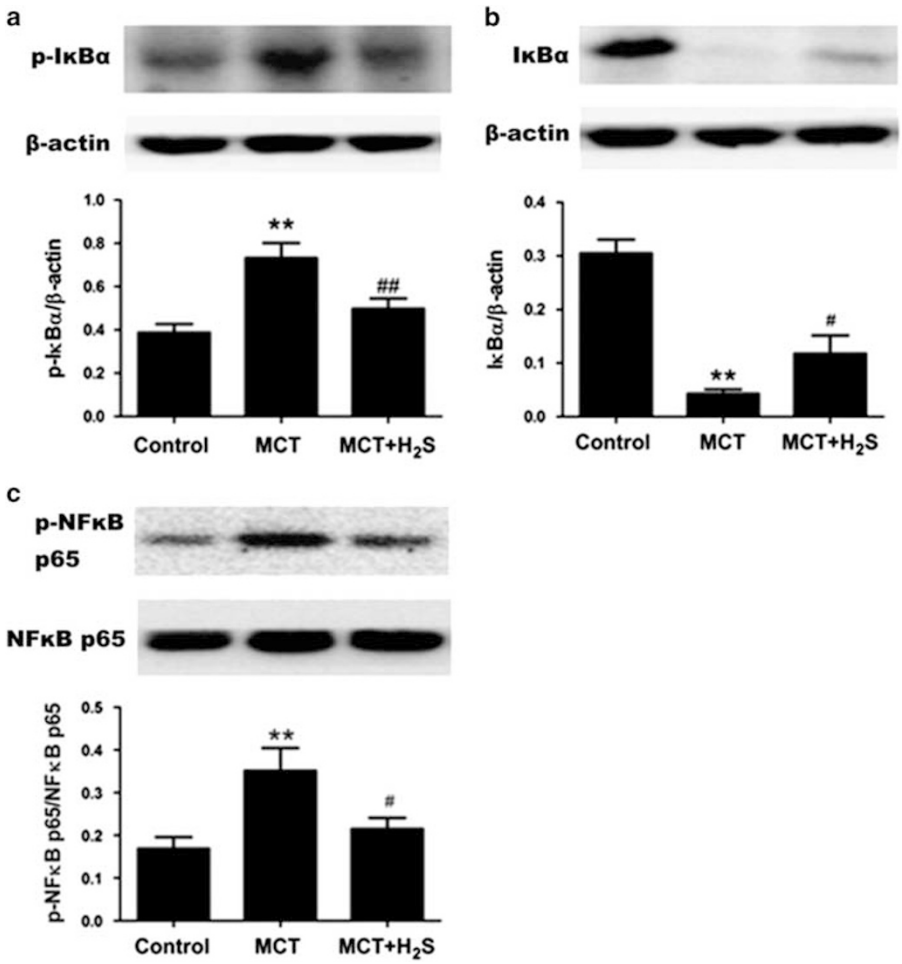

Figure 4 H_2S inhibited NF-κB activation in rats with MCT-induced pulmonary hypertension. (**a**) Western blotting analysis of IκBα phosphorylation in rat lung tissues; (**b**) Western blotting analysis of IκBα expression in rat lung tissues; (**c**): Western blotting analysis of NF-κB phosphorylation in rat lung tissues. Note: mean ± s.e., $n=8$ of each group, $^{**}P<0.01$ *vs.* Control group, $^{\#\#}P<0.01$, $^{\#}P<0.05$ *vs.* MCT group. H_2S, hydrogen sulfide; MCT, monocrotaline.

($P<0.01$), and NF-κB p65 phosphorylation was upregulated significantly ($P<0.01$) in MCT rat lung tissue. However, in the MCT+H_2S group compared with the MCT group, the level of phosphorylated IκBα was reduced ($P<0.01$), the level of total IκBα increased ($P<0.05$), and NF-κB p65 phosphorylation decreased significantly ($P<0.05$; Figure 4).

The Endogenous H_2S Pathway was Downregulated in MCTP-Stimulated HPAECs

Compared with the control cells, the H_2S content in supernatant of the MCTP cells was reduced ($P<0.01$) and the fluorescent intensity of the H_2S probe was also weak in MCTP-treated cells. In addition, CSE levels were decreased ($P<0.01$) in MCTP-treated cells. After treatment with H_2S, H_2S synthesis and CSE levels increased (both $P<0.05$), with augmented fluorescent intensity of the H_2S probe (Figure 5).

H_2S Protected Against Inflammation in MCTP-Stimulated HPAECs

ICAM-1 expression in HPAECs was increased compared with the control cells ($P<0.01$). By contrast, ICAM-1 expression in the MCTP+H_2S cells decreased compared with the MCTP-treated cells ($P<0.01$; Figure 6).

H_2S Inhibited NF-κB Activation in MCTP-Stimulated HPAECs

Western blotting and confocal microscopy demonstrated that the NF-κB p65 nuclear translocation in MCTP-treated cells was increased compared with the control cells ($P<0.01$). However, compared with the MCTP-treated cells, the NF-κB p65 nuclear translocation in the MCTP+H_2S -treated cells was decreased ($P<0.01$; Figures 7a–c).

EMSA and AM-ELISA revealed that, compared with the control cells, the MCTP-treated cells showed significantly increased NF-κB p65 DNA-binding activity ($P<0.01$);

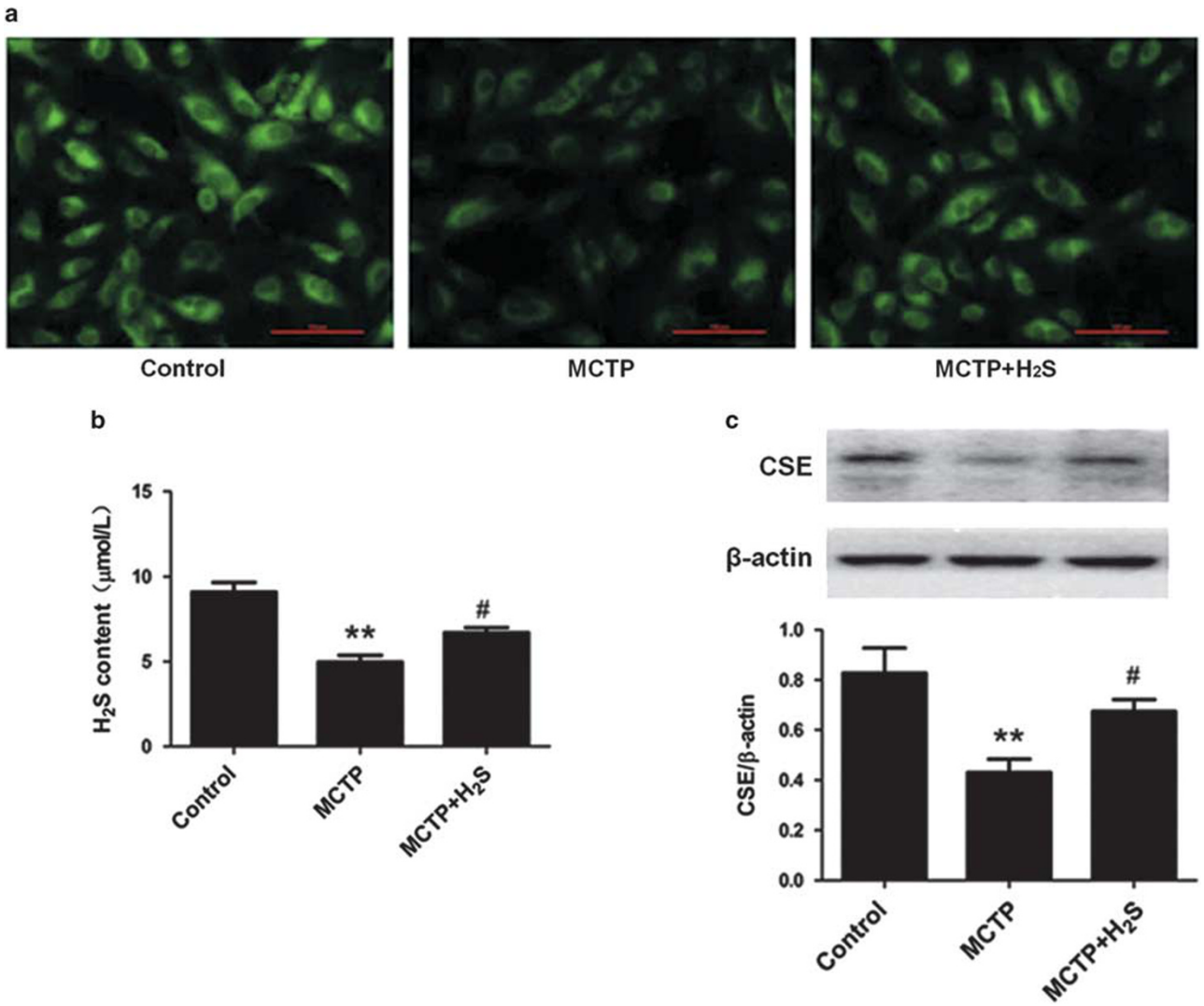

Figure 5 The endogenous H_2S pathway was downregulated in MCTP-stimulated HPAECs. (**a** and **b**) H_2S generation in HPAECs. H_2S localization detected by fluorescent probe (**a**), green fluorescence indicates H_2S, ×400; H_2S content analyzed by free radical detector (**b**); (**c**) CSE protein expression in HPAEC. Note: mean ± s.e., all data obtained by three independent experiments, $^{**}P<0.01$ *vs.* Control group, $^{\#}P<0.05$ *vs.* MCTP group. CSE, cystathionine-γ-lyase; HPAEC, human pulmonary arterial endothelial cell; H_2S, hydrogen sulfide; MCT, monocrotaline; MCTP, MCT pyrrole.

however, the NF-κB p65 DNA-binding activity was significantly decreased ($P<0.01$) in the MCTP+H_2S cells compared with the MCTP cells (Figures 7d and e).

DISCUSSION

Increasing evidence suggests that the inflammatory response, particularly vascular inflammation, is an important initial step in pulmonary vascular remodeling and PAH, which is closely associated with pulmonary hypertension development and outcome. Several studies have explored the pathogenesis of vascular inflammation in PAH, although the details of the synthesis and release of inflammatory cytokines in pulmonary arterial endothelial cells and the regulatory mechanism for pulmonary vascular inflammation remain incompletely understood.[20,21]

H_2S, mainly produced by CSE in the cardiovascular system of mammals, relaxes blood vessels, lowers blood pressure, and inhibits vascular smooth muscle cell proliferation, establishing its crucial role in cardiovascular regulation.[22,23] Previous studies showed that H_2S could attenuate pulmonary vascular remodeling and pulmonary hypertension by inhibiting pulmonary artery smooth muscle cell proliferation, as well as promoting apoptosis of pulmonary arterial smooth muscle and pulmonary vascular collagen. Du *et al.*[13] found that H_2S could inhibit the inflammatory response stimulated by ox-LDL in monocyte macrophages. H_2S also had a role in inhibiting vascular inflammation in Apo $E^{-/-}$ mice with atherosclerosis.[10] However, whether H_2S protects against PAH by inhibiting pulmonary vascular inflammation requires further study.

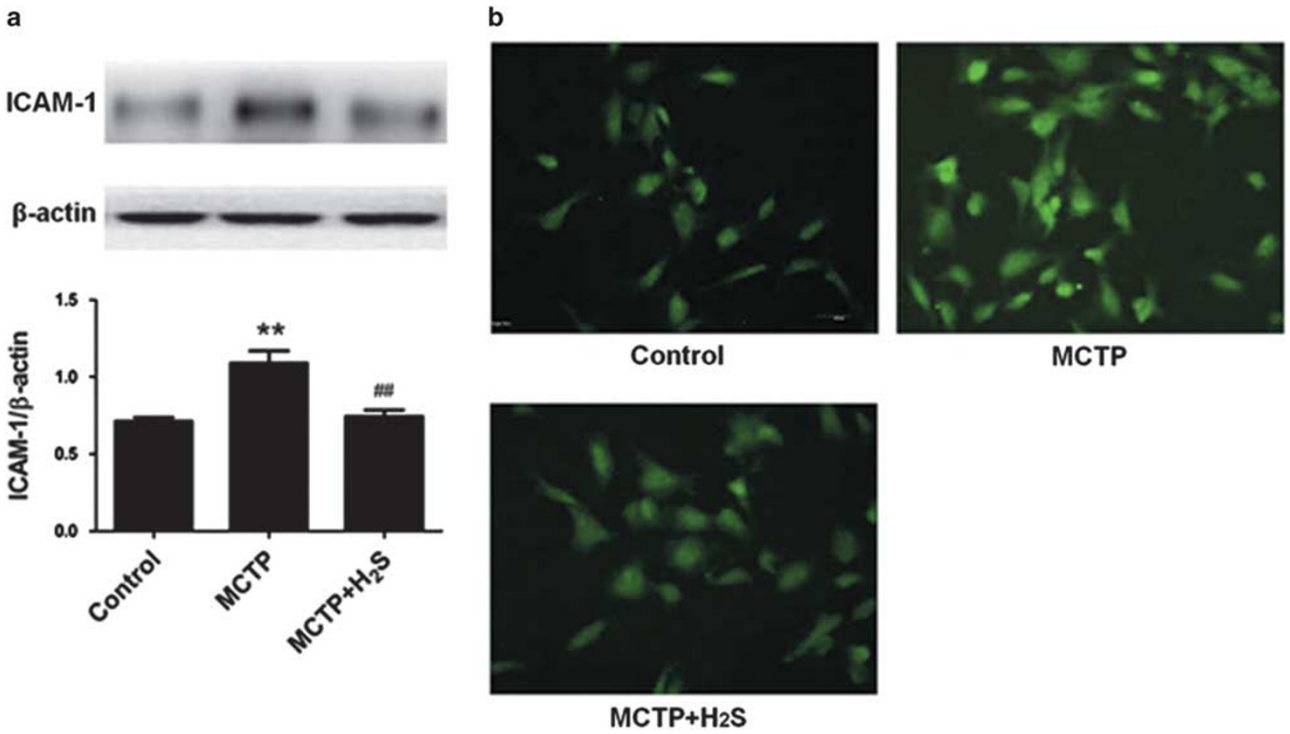

Figure 6 H_2S protected against inflammation in MCTP-stimulated HPAECs. (**a**) ICAM-1 expression in HPAECs determined by western blotting; (**b**) ICAM-1 expression in HPAECs examined by immunoflluorescent imaging. Note: mean ± s.e., three independent experiments. $^{**}P<0.01$ *vs.* Control group, $^{\#\#}P<0.01$ *vs.* MCTP group. HPAEC, human pulmonary arterial endothelial cell; H_2S, hydrogen sulfide; MCT, monocrotaline; MCTP, MCT pyrrole.

In the present study, we used MCT to build a rat model of PAH with pulmonary vascular inflammation. We also used MCTP, the active form of MCT, to produce a cell model of pulmonary vascular endothelial injury with vascular inflammation. We first examined the changes in endogenous H_2S/CSE in pulmonary arterial endothelial cells. The MCT-treated rats displayed increased pulmonary arterial pressure, significant right ventricular hypertrophy, elevated RMT and RMA of the pulmonary vasculature, and increased inflammatory cytokine levels in rat plasma and lung tissue. In the MCT-treated rats, the endogenous H_2S content in rat plasma and lung tissue were decreased and CSE expression in lung tissue was significantly reduced. *In vitro* studies showed that there was a significant increase in ICAM-1 expression in the MCTP group, whereas endogenous H_2S synthesis and CSE expression decreased. These results suggested that the endogenous H_2S/CSE pathway was significantly downregulated in the development of pulmonary vascular inflammation and pulmonary hypertension stimulated by MCT.

To further investigate the regulatory role of H_2S in pulmonary vascular inflammation and PAH, we investigated the effect of an H_2S donor on pulmonary vascular inflammation. *In vivo,* the H_2S donor reduced pulmonary arterial pressure, lowered the RMT and RMA of pulmonary vasculature, and reduced inflammatory cytokine levels in plasma and lung tissues of MCT+H_2S rats compared with MCT-treated rats. *In vitro*, we observed a marked decrease in ICAM-1 expression in MCTP+H_2S-treated HPAECs compared with MCTP-treated HPAECs. Therefore, we concluded that H_2S could protect against pulmonary vascular inflammation and thus antagonize pulmonary hypertension, although the molecular mechanism remains to be investigated.

The NF-κB pathway is the canonical process of the inflammatory response. In the resting state, p65/p50 and IκBα form a trimer in the cytoplasm. Inflammatory stimuli induce IκBα phosphorylation, ubiquitination, and degradation, thus releasing NF-κB p65/p50 from the trimer. p65/p50 is then transferred into the nucleus to regulate the expression of inflammation-related genes.[24] In the present study, we targeted the NF-κB pathway to explore the signal transduction mechanisms underlying the inhibitory effect of H_2S on pulmonary vascular inflammation in MCT-induced PAH rats and MCTP-stimulated HPAECs. Animal experiments showed that IκBα phosphorylation was enhanced, whereas the IκBα content decreased; however, NF-κB p65 phosphorylation was increased in MCT rat lung tissue. After H_2S administration, IκBα phosphorylation was reduced, whereas the IκBα content increased; however, NF-κB p65 phosphorylation was decreased in lung tissues. Cellular experiments showed that MCTP promoted the nuclear translocation of NF-κB p65 and enhanced NF-κB p65 DNA-binding activity. However, treatment with H_2S inhibited NF-κB p65 nuclear translocation and the DNA-binding activity in MCTP-stimulated cells. These results suggested that H_2S protected against pulmonary

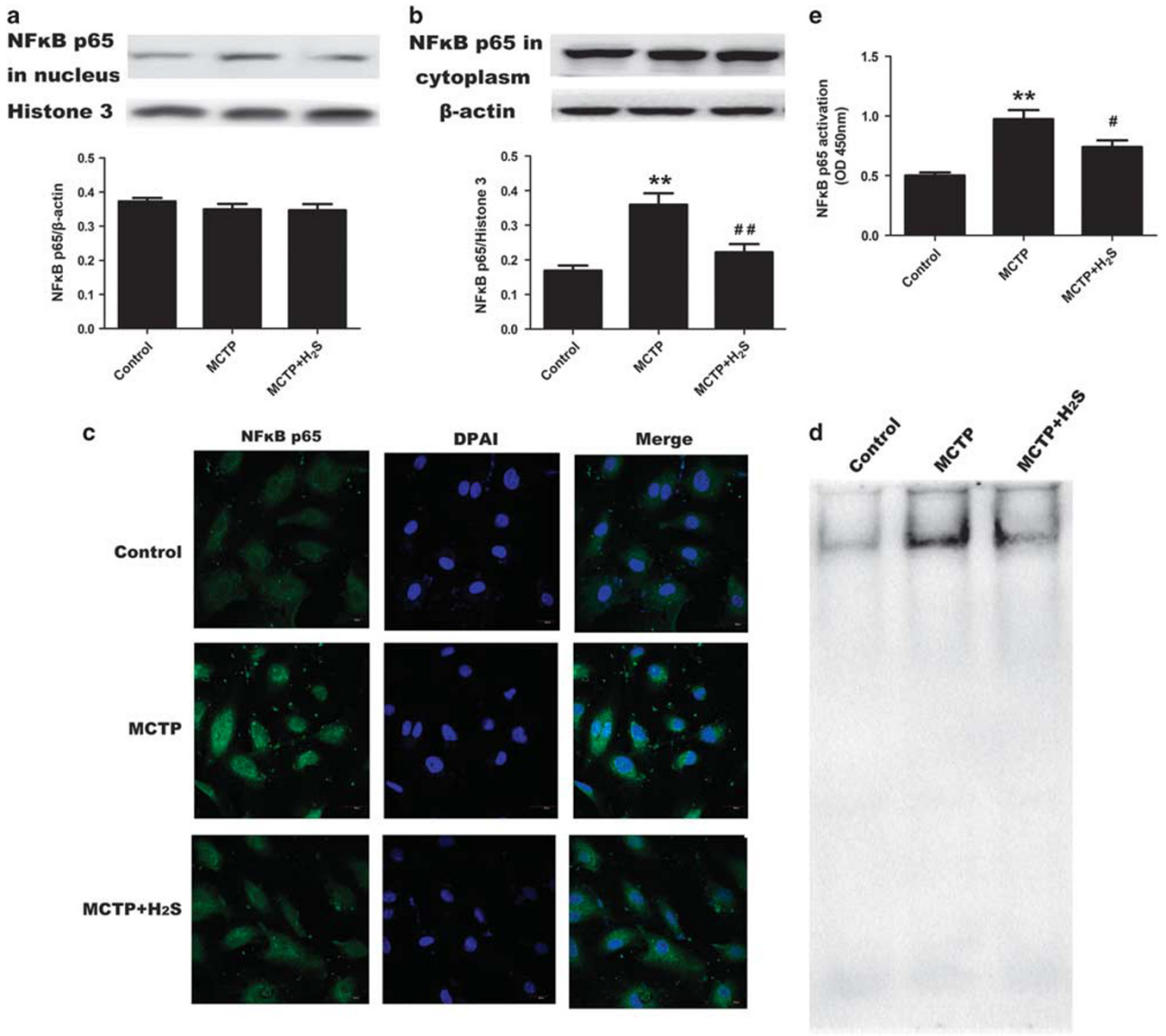

Figure 7 H_2S inhibited NF-κB activation in MCTP-stimulated HPAECs. (**a** and **b**) Western blotting analysis of NF-κB p65 in nucleus (**a**) and cytoplasm (**b**) of HPAECs; (**c**) NF-κB p65 nuclear translocation in HPAECs examined by confocal laser scanning. (**d**) NF-κB p65 DNA-binding activity of HPAECs determined by EMSA; (**e**) NF-κB p65 DNA-binding activity of HPAECs determined by AM-ELISA. Note: mean ± s.e., three independent experiments, $^{**}P<0.01$ *vs.* Control group, $^{\#\#}P<0.01$ *vs.* MCTP group. ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; HPAEC, human pulmonary arterial endothelial cell; H_2S, hydrogen sulfide; MCT, monocrotaline; MCTP, MCT pyrrole.

vascular inflammation by attenuating the NF-κB signaling pathway.

In summary, our results revealed a marked downregulation of endogenous H_2S synthesis in pulmonary artery endothelial cells of rats with MCT-induced PH. H_2S could reduce pulmonary vascular pressure, relieve pulmonary vascular remodeling, inhibit pulmonary vascular endothelial cellular inflammation, and attenuate the NF-κB signaling pathway in pulmonary arterial endothelial cells. Our data suggested that downregulation of the endogenous H_2S pathway was an important mechanism involved in the pathogenesis and regulation of pulmonary vascular inflammation in pulmonary hypertensive rats. H_2S could inhibit the inflammatory response of pulmonary arterial endothelial cells in MCT-induced PAH, probably via a mechanism related to NF-κB signaling pathway attenuation. The present study increases our knowledge of the pathogenesis of pulmonary vascular inflammation in PAH, thus providing potential novel targets for the anti-inflammatory treatment of PAH.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Tuder RM, Stacher E, Robinson J, *et al.* Pathology of pulmonary hypertension. Clin Chest Med 2013;34:639–650.
2. Price LC, Wort SJ, Perros F, *et al.* Inflammation in pulmonary arterial hypertension. CHEST J 2012;141:210–221.
3. Le Hiress M, Tu L, Ricard N, *et al.* Proinflammatory signature of the dysfunctional endothelium in pulmonary hypertension. Role of the macrophage migration inhibitory factor/CD74 complex. Am J Respir Crit Care Med 2015;192:983–997.
4. Budhiraja R, Tuder RM, Hassoun PM. Endothelial dysfunction in pulmonary hypertension. Circulation 2004;109:159–165.
5. Price LC, Montani D, Tcherakian C, *et al.* Dexamethasone reverses monocro-taline-induced pulmonary arterial hypertension in rats. Eur Respir J 2011;37:813–822.
6. Wu D, Hu Q, Zhu Y. Therapeutic application of hydrogen sulfide donors: the potential and challenges. Front Med 2016;10:18–27.
7. Tang C, Li X, Du J. Hydrogen sulfide as a new endogenous gaseous transmitter in the cardiovascular system. Curr Vasc Pharmacol 2006;4: 17–22.
8. Jin HF, Liang C, Liang JM, *et al.* Effects of hydrogen sulfide on vascular inflammation in pulmonary hypertension induced by high pulmonary blood flow: experiment with rats. Zhonghua Yi Xue Za Zhi 2008;88: 2235–2239.
9. Tao BB, Cai WJ, Zhu YC. H_2S is a promoter of angiogenesis: identification of H_2S "Receptors" and its molecular switches in vascular endothelial cells[M]. Handb Exp Pharmacol 2015;230: 137–152.
10. Wang Y, Zhao X, Jin H, *et al.* Role of hydrogen sulfide in the development of atherosclerotic lesions in apolipoprotein E knockout mice. Arterioscler Thromb Vasc Biol 2009;29:173–179.
11. Chen F, Castranova V, Shi X. New insights into the role of nuclear factor-κB in cell growth regulation. Am J Pathol 2001;159: 387–397.
12. Dep Prete A, Allavena P, Santoro G, *et al.* Molecular pathways in cancer-related inflammation. Biochem Med (Zagreb) 2011;21:264–275.
13. Du J, Huang Y, Yan H, *et al.* Hydrogen sulfide suppresses oxidized low-density lipoprotein (ox-LDL)-stimulated monocyte chemoattractant protein 1 generation from macrophages via the nuclear factor κB (NF-κB) pathway. J Biol Chem 2014;289:9741–9753.
14. Maarman G, Lecour S, Butrous G, *et al.* A comprehensive review: the evolution of animal models in pulmonary hypertension research; are we there yet?. Pulm Circ 2013;3:739.
15. Gomez-Arroyo JG, Farkas L, Alhussaini AA, *et al.* The monocrotaline model of pulmonary hypertension in perspective. Am J Physiol Lung Cell Mol Physiol 2012;302:L363–L369.
16. Ramos M, Lamé MW, Segall HJ, *et al.* Monocrotaline pyrrole induces Smad nuclear accumulation and altered signaling expression in human pulmonary arterial endothelial cells. Vasc Pharmacol 2007;46:439–448.
17. Sun Y, Tian Y, Prabha M, *et al.* Effects of sulfur dioxide on hypoxic pulmonary vascular structural remodeling. Lab Invest 2010;90: 68–82.
18. Huang P, Chen S, Wang Y, *et al.* Down-regulated CBS/H_2S pathway is involved in high-salt-induced hypertension in Dahl rats. Nitric Oxide 2015;46:192–203.
19. Zhu M, Du J, Liu AD, *et al.* L-cystathionine inhibits oxidized low density lipoprotein-induced THP-1-derived macrophage inflammatory cytokine monocyte chemoattractant protein-1 generation via the NF-κB pathway. Sci Rep 2015;5:10453.
20. Voelkel NF, Cool C. Pathology of pulmonary hypertension. Cardiol Clin 2004;22:343–351.
21. Guignabert C, Tu L, Girerd B, *et al.* New molecular targets of pulmonary vascular remodeling in pulmonary arterial hypertension: importance of endothelial communication. Chest J 2015;147:529–537.
22. Kolesnikov SI, Vlasov BY, Kolesnikova LI. Hydrogen sulfide as a third essential gas molecule in living tissues. Vestn Ross Akad Med Nauk 2015;2:237–241.
23. Jin HF, DU JB, Tang CS. Waste gas is not waste: advance in the research of hydrogen sulfide. Sheng Li Xue Bao 2010;62:495–504.
24. Czyz M. Specificity and selectivity of the NF-kappa B response. Postepy Biochem 2004;51:60–68.