Interventions and mechanisms of N-acetylcysteine on monocrotaline-induced pulmonary arterial hypertension

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Abstract. The aim of the present study was to investigate the impact of N-acetylcysteine (NAC) on the expression of activin receptor-like kinase-1 (ALK-1) and mothers against decapentaplegic homolog 1 (Smad1) in the pulmonary artery of rats with pulmonary arterial hypertension (PAH), and to explore the possible mechanisms underlying its effects on pulmonary vascular remodeling (PVR). In total, 32 Wistar rats were randomly divided into four groups: Control, model, low-dose (100 mg/kg/day) NAC and high-dose (500 mg/kg/day) NAC. Monocrotaline (MCT) was intraperitoneally injected to prepare the model, and the right ventricular hypertrophy index (RVHI) and hemodynamic parameters were detected 6 weeks later. Hematoxylin and eosin staining was used to observe the pulmonary arterial structural changes and evaluate the peri-pulmonary artery inflammation score. Additionally, western blot analysis was used to detect the protein expression of ALK-1 and Smad1 in the pulmonary artery. The results demonstrated that treatment with NAC reduced RVHI and mean pulmonary artery pressure. In addition, NAC reduced the MCT-induced PVR, pulmonary inflammation score and upregulation of ALK-1 and Smad1. These results indicate that ALK-1 and Smad1 participate in the formation of PAH and the process of PVR, and suggest that NAC may inhibit PAH by inhibiting the expression of ALK-1 and Smad1 in the pulmonary artery.

Introduction

Pulmonary arterial hypertension (PAH) is a disease with poor prognosis and high mortality rate, and is defined as a mean pulmonary arterial pressure (mPAP) of ≥25 mmHg in the resting state and ≥30 mmHg when moving. Its main feature is progressive pulmonary occlusion resulting in gradually increased pulmonary vascular resistance and pulmonary arterial pressure, accompanied by irreversible pulmonary vascular remodeling (PVR) and the occurrence of right-sided heart failure (1). Therefore, PAH is harmful to patients. The survival time of idiopathic PAH following diagnosis is only 2.5-3.4 years (2), and the condition currently has no effective treatment.

The pathogenesis of PAH is unclear; however, it appears to involve PVR on a genetic and immunological basis (3,4). Cells, circulatory mediators and molecular genetics have an involvement in the origination and development of PAH; currently, activin receptor-like kinase 1 (ALK-1) is considered to be closely associated with idiopathic PAH, and its role in the pathogenesis of PAH has attracted growing attention (5-7). The ALK-1 gene is expressed in endothelial, smooth muscle, hepatic stellate and cartilage cells, fibroblasts, monocytes and macrophages, and is critical in the regulation of developmental and pathological angiogenesis angiogenesis (8,9). The over-expression of ALK-1 leads to signaling abnormalities, thus causing changes in the growth state of endothelial and smooth muscle cells, followed by PVR and PAH (9-11).

The glutathione precursor N-acetylcysteine (NAC) may be used to treat pulmonary fibrosis, inhibit the proliferation, adhesion and migration of endothelial cells, reduce the transforming growth factor-β (TGF-β)-induced production of collagens, regulate gene expression and signal transduction, improve endothelial functions, attenuate inflammation and oxidation, and regulate the immune system, and thus exhibits great significance in the reversal of PVR (12,13). A previous study has revealed that NAC is able to ameliorate PAH and right ventricular functions through immunoregulatory mechanisms (14).

The present study used a monocrotaline (MCT)-induced rat PAH model to observe the protein expression of ALK-1 and mothers against decapentaplegic homolog 1 (Smad1) in the ALK-1 signal transduction pathway, with the aim of clarifying their roles in PAH and PVR. The model was also used to investigate the pathological changes of the pulmonary artery walls in rats following NAC intervention, and the changes of mean right ventricular pressure (mRVP), mPAP, weight of the right ventricle (RV), weight of the left ventricle and septum (LV+S), and the right ventricular hypertrophy index (RVHI). In addition, the association of NAC treatment with changes

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of the ALK-1 signaling pathway was investigated. The results may provide new therapeutic rationale for the treatment of PAH, and provide experimental evidence for the development of effective treatment protocols.

Materials and methods

Animals and model preparation. In total, 32 male Wistar rats (age, 8 weeks; weight, 180-200 g; License No. scxk-Lu-20130001; Shandong Lukang Pharmaceutical Group Ltd., Jining, China) were housed in the Tianjin Experimental Animal Center. The rats were given free access to food and water at a constant temperature of 22±2˚C and humidity of 55±5%, with natural light for 1 week of adaptive feeding prior to the experiment. The rats were randomly divided into four groups (each n=8): Control (group C), model (group M), low-dose NAC (group N1) and high-dose NAC (group N2). In the M, N1 and N2 groups, each rat was intraperitoneally injected with 60 mg/kg MCT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for modeling, and the rats in group C were intraperitoneally injected with an equal amount of normal saline (NS). From the modeling day, the rats were orally administered NS (groups C and M), 100 mg/kg/day NAC (group N1) or 500 mg/kg/day NAC (group N2) under the same feeding conditions for 6 weeks. A right-heart catheter and physiological monitoring system (PowerLab; ADInstruments, Sydney, Australia) were used to detect the right ventricular systolic pressure and mPAP of each group. Following that, the animals were sacrificed, the chest was opened and the heart and lungs were excised to observe and compare the RV and LV+S weights and to determine the RVHI. The right lower lung tissue was then sampled, and fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining. One ~1-cm section of pulmonary artery was sampled 3 mm proximal to the lung hilum, washed with PBS and then stored at -80˚C for detection of the protein expression of ALK-1 and Smad1 using western blotting. The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Qingdao University (Qingdao, China). Ethical approval was obtained from the Ethics Committee of Qingdao University Hospital.

Detection of artery pressure and RVHI. Following anesthetization with an intraperitoneal injection of chloral hydrate (400 mg/kg), the hemodynamics of each rat were detected as previously described (15,16), and mPAP and mRVP were determined using right-heart catheterization and the PowerLab physiological monitoring system. The animals were then sacrificed, and the whole heart was removed. The RV and LV+S along the interventricular septum were then isolated and weighed, and the RVHI was calculated using the following formula: RVHI=RV/(LV+S).

H&E staining. Following sacrifice, the rat chest was surgically opened for excision of the heart and lungs. The right lung was fixed for 30 min via the intratracheal perfusion of 4% paraformaldehyde in order to flatten and smooth the lung surface. The right hilum was horizontally sliced to sample the right lower lung tissue, which was then fixed in 4% paraformaldehyde at 20˚C for 36-48 h, paraffin-embedded, sliced into 3-μm-thick sections and stained with H&E at 20˚C for 20 min. In each slice, 10 small arteries (50-100 μm in diameter) were randomly selected for measurement of their wall thickness (WT), external diameter (ED), mean total area (TA) and internal area (IA) using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). WT% and wall area (WA)% were then calculated in order to evaluate PVR as follows: WT%=(2 x WT/ED) x 100; WA%=(TA-IA)/TA x 100, and the mean values were determined. In addition, in each slice, 10 small pulmonary arteries (50-100 μm in diameter) were selected for the observation of peri-pulmonary artery inflammatory infiltration under a light microscope at a magnification of x400. The conditions were then scored according to the degree of infiltration of peri-pulmonary vascular inflammatory cells as follows: i) No infiltration, 0 points; ii) mild infiltration, 1 point; iii) moderate infiltration, 2 points; iv) severe infiltration, 3 points and v) very severe infiltration, 4 points.

Western blot analysis. Following sacrifice, a ~1-cm section of pulmonary artery was sampled 3 mm proximal to the lung hilum. The surrounding connective and fat tissues were removed, and the remaining artery was washed with PBS and stored at -80˚C. Radioimmunoprecipitation assay lysis buffer (cat. no. CW2333S; Beijing ComWin Biotech Co., Ltd., Beijing, China) was added to the pulmonary artery incubated for 20 min on the ice. The protein concentration was then detected using the bicinchoninic acid assay method. A sample containing 20 μg protein/lane was gently mixed with 100 ml/l SDS-PAGE loading buffer for 10 min denaturation at 95˚C, and then subjected to electrophoresis in a 10% SDS-PAGE. The isolated protein bands were transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA), which was placed into a bovine serum albumin blocking buffer (cat. no. CW0054; Beijing ComWin Biotech Co., Ltd.) at room temperature, and shaken slowly for 1 h. Rabbit anti-mouse ALK-1 (cat. no. sc-19547), rabbit anti-mouse Smad1 polyclonal (cat. no. sc-7965) and β-actin (cat. no. sc-47778; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies were then applied to uniformly cover the membrane surface, and incubated overnight at 4˚C. Following repeated washing of the membrane, the horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:3,000; cat. no. 31460; Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added in for 1 h incubation at room temperature, followed by washing with TBST, and cECL Western Blot kit (cat. no. CW0048; Beijing ComWin Biotech Co., Ltd.). Labworks 4.0 software (UVP, LLC, Phoenix, AZ, USA) was used to scan the film and determine the grayscale values, with β-actin (43 kDa) serving as the internal control, and to calculate the expression levels of the proteins.

Statistical analysis. Measurement data are expressed as the mean ± standard deviation. Differences among the groups were analyzed using analysis of variance followed by Tukey's test. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used to conduct the data analysis.

Results

General conditions and survival. The rats in group M exhibited a slow increase in body mass, slow actions, dull fur and reduced food intake compared with those in group C. Furthermore, they demonstrated shortness of breath and a significantly reduced body weight at the end of the 6-week experimental period (P<0.05). The rats in groups N1 and N2 exhibited improved activity, fur condition, food intake and breathing compared with those in group M; however, their body weights exhibited no significant difference compared with those in group M (P>0.05). The survival rates in groups C, M, N1 and N2 were 100, 75, 88 and 100%, respectively, and the survival rates for groups N1 and N2 were significantly higher compared with those in group M (P<0.05; Table I).

Hemodynamics and right ventricular remodeling. At the end of the 6 weeks, mRVP, mPAP and RVHI in group M were significantly higher compared with those in group C (P<0.01). Furthermore, mRVP, mPAP and RVHI in groups N1 and N2 were significantly reduced compared with those in group M (P<0.05) but were significantly higher compared with those in group C (P<0.05). The RVHI in group N2 was significantly reduced compared with that in group N1 (P<0.05) but mRVP and mPAP exhibited no significant difference between groups N1 and N2 (P>0.05), as depicted in Table II and Fig. 1.

Morphological changes of pulmonary arteries. Respiratory bronchioles (ED, 50-100 μm) or small pulmonary arteries with integral structures accompanied by alveoli were sampled for observation. H&E staining revealed that the pulmonary arterial walls in group C were thin, with continuous endothelial cells, and no cell shedding or necrosis, or luminal stenosis. Furthermore, no inflammatory cell infiltration was observed in and around the pulmonary arteries. Compared with group C, the pulmonary arterial walls in group M were thickened, with proliferation and thickening of the smooth muscle. In addition, the lumen exhibited stenosis, and degeneration, swelling, necrosis and shedding of the endothelial cells were observed. Furthermore, numerous monocytes and neutrophils infiltrated the pulmonary arterial wall and the surroundings. The WT%, WA% and inflammation score were significantly increased in group M compared with group C (P<0.01). Compared with group M, groups N1 and N2 demonstrated significant attenuation of the changes in pulmonary artery WT and stenosis, and the WT%, WA% and inflammation scores were significantly reduced (P<0.05). Furthermore, significant differences in these three variables were detected between groups N1 and N2 (P<0.05). However, the WT% and WA% values for rats in groups N1 and N2 remained higher compared with those in group C (P<0.05), as demonstrated in Fig. 2 and Table III.

Western blotting. The western blotting results revealed that the protein expression levels of ALK-1 and Smad1 in group M were significantly increased compared with those in group C (P<0.01). Additionally, the protein expression levels of ALK-1 and Smad1 in groups N1 and N2 were significantly decreased compared with those in group M (P<0.05), and the intergroup difference was significant (P<0.05). However, the expression levels in groups N1 and N2 remained higher than those in the control group (P<0.01) as shown in Fig. 3 and Table IV.

Table I. Body weights and survival rates of rats in each group.

Groups	n	Survival rate (%)	Week 0 (g)	Week 6 (g)
C	8	100	183.92±10.32	293.57±16.48
M	6	75[a]	183.85±11.37	264.34±12.78[a]
N1	7	88[a,b]	186.59±13.78	267.81±14.73[a]
N2	8	100[c]	185.82±9.56	271.75±12.94[a]

Data are presented as mean ± standard deviation. [a]P<0.05 vs. group C; [b]P<0.05 and [c]P<0.01 vs. group M. C, normal control; M, model of pulmonary arterial hypertension; N1, low-dose N-acetylcysteine; N2, high-dose N-acetylcysteine.

Table II. Hemodynamics and right ventricle indices at 6 weeks after the monocrotaline injection.

Group	n	mRVP (mmHg)	mPAP (mmHg)	RVHI
C	8	20.78±1.85	21.37±1.73	0.22±0.01
M	6	45.29±2.79[a]	49.80±2.96[a]	0.88±0.03[a]
N1	7	34.25±2.25[b,c]	31.93±1.87[b,c]	0.52±0.02[a,c]
N2	8	33.18±2.42[b,c]	27.03±1.80[b,c]	0.30±0.01[b,d,e]

Data are presented as mean ± standard deviation. [a]P<0.01 and [b]P<0.05 vs. group C; [c]P<0.05 and [d]P<0.01 vs. group M; [e]P<0.05 vs. group N1. C, normal control; M, model of pulmonary arterial hypertension; N1, low-dose N-acetylcysteine; N2, high-dose N-acetylcysteine; mRVP, mean right ventricular pressure; mPAP, mean pulmonary arterial pressure; RVHI, right ventricular hypertrophy index.

Discussion

PAH is a progressive disease in which the pulmonary artery pressure progressively increases, eventually leading to progressive right-heart failure and mortality. The main pathological feature of PAH is irreversible pulmonary arterial remodeling. Additionally, progressive occlusion gradually increases the pulmonary vascular resistance and pulmonary artery pressure, which is accompanied by irreversible PVR and the occurrence of right-heart failure (17). The causes of PAH are complex, and the development of this disease is a multifactorial process; however, recent studies conducted on the pathogenesis of PAH have made considerable progress (18-20). Genetic mutation of bone morphogenetic protein receptor 2 has been identified in patients with familial PAH (21). Other pathophysiological changes that have been identified for PAH include functional abnormalities of pulmonary vascular endothelial cells, K^+ channel lesions on the cell membrane of pulmonary vascular smooth muscles, changes to the roles of 5-hydroxytryptamine transporters, and increased matrix synthesis in adventitia (1,22). However, the pathogenesis of PAH remains incompletely elucidated. As mentioned above, the ALK-1 gene is closely associated with

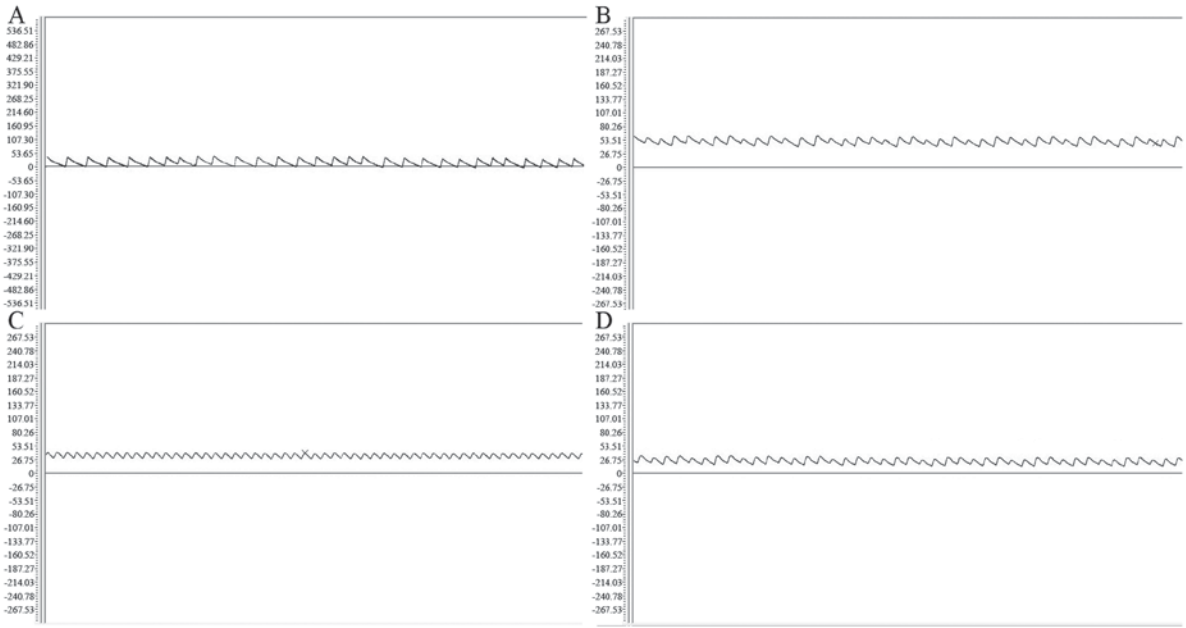

Figure 1. Oscillograms of mean right ventricular pressure and mean pulmonary arterial pressure of each group at the end of 6 weeks (A) normal control (B) model of pulmonary arterial hypertension; (C) low-dose N-acetylcysteine; (D) high-dose N-acetylcysteine.

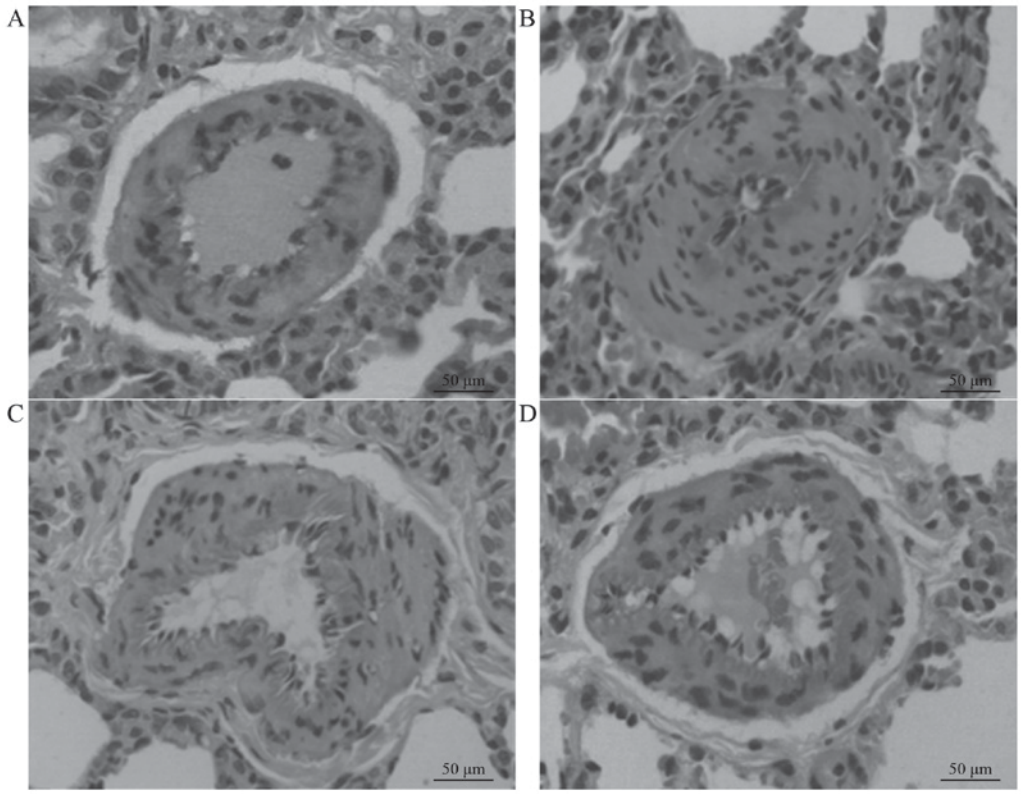

Figure 2. Hematoxylin and eosin staining results of pulmonary artery sections in each group (magnification, x400). (A) normal control (B) model of pulmonary arterial hypertension; (C) low-dose N-acetylcysteine; (D) high-dose N-acetylcysteine.

idiopathic PAH, and the present study used NAC for therapeutic intervention in a rat model of PAH, with the aim of observing its effects on the protein expression of ALK-1 and Smad1 and the pathological changes of the pulmonary artery wall. Additionally, changes of mRVP, mPAP, RV and LV+S weights and the RVHI were also observed in order to explore whether treatment with NAC was associated with changes in the ALK-1 signaling pathway.

ALK-1 is a type I receptor of the TGF-β superfamily on the endothelial cell surface, which is able to regulate cell proliferation, migration and the expression of extracellular matrix proteins (23). TGF-β is distributed in a variety of

Table III. Pulmonary artery and inflammatory indices in each group at 6 weeks after the monocrotaline injection.

Group	n	WT%	WA%	Inflammation score (points)
C	8	24.67±4.21	38.65±3.57	0.74±0.03
M	6	60.93±6.58[a]	75.84±5.83[a]	3.41±0.68[a]
N1	7	48.37±5.21[a,b]	61.77±5.58[a,b]	2.03±0.81[a,c]
N2	8	40.65±5.12[c-e]	50.63±4.26[c-e]	0.82±0.02[c,f]

Data are presented as mean ± standard deviation. [a]P<0.01 vs. group C; [b]P<0.05 and [c]P<0.01 vs. group M; [d]P<0.05 vs. group C; [e]P<0.05 and [f]P<0.01 vs. group N1. WT, wall thickness; C, normal control; M, model of pulmonary arterial hypertension; N1, low-dose N-acetylcysteine; N2, high-dose N-acetylcysteine; WT%, pulmonary vascular wall thickness as a percentage of external diameter; and WA%, wall area as a percentage of vascular area.

Table IV. Comparison of ALK-1 and Smad1 protein expression among different groups.

Group	ALK-1	Smad1
C	0.1044±0.021	0.1735±0.034
M	0.4280±0.073[a]	0.4504±0.082[a]
N1	0.3175±0.086[a,b]	0.3524±0.078[a,b]
N2	0.2275±0.056[a,c,d]	0.2777±0.065[a,c,d]

Data are presented as mean ± standard deviation. [a]P<0.01 vs. group C; [b]P<0.05 and [c]P<0.01 vs. group M; [d]P<0.05 vs. group N1. C, normal control; M, model of pulmonary arterial hypertension; N1, low-dose N-acetylcysteine; N2, high-dose N-acetylcysteine; ALK-1, activin receptor-like kinase 1; Smad1, mothers against decapentaplegic homolog 1.

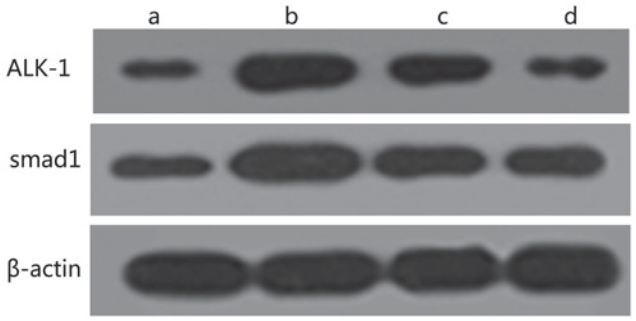

Figure 3. Western blot results showing ALK-1 and Smad1 expression in each group. Lane a, control; lane b, model of pulmonary arterial hypertension; lane c, low-dose N-acetylcysteine; lane d, high-dose N-acetylcysteine. ALK-1, activin receptor-like kinase 1; Smad1, mothers against decapentaplegic homolog 1.

tissues and cells in the human body, serving important roles in the regulation of cell proliferation and differentiation, extracellular matrix synthesis and angiogenesis (10). It has been suggested that as the type I receptor of TGF-β, ALK-5, activates the Smad2/3 pathway in endothelial cells and another TGF-β type I receptor, ALK-1, activates the Smad1/5 pathway in endothelial cells, the interaction of ALK-1 with Smad1/5 should promote the proliferation and migration of endothelial cells (the active phase of angiogenesis), whereas the interaction of ALK-5 with Smad2/3 should inhibit this process, which is the regression stage of angiogenesis (24). TGF-β regulates endothelial cell functions via interactions with ALK-1 and ALK-5, and the overexpression of ALK-1 reduces the signaling conduction of ALK-5 in the extracellular matrix. However, the excessive expression of ALK-5 increases the signal transduction of ALK-1 in the extracellular matrix (25).

Therefore, it may be speculated that the balance between ALK-1/TGF-β/ALK-5 interactions is an important signal transduction pathway in the angiogenesis process, and that abnormalities of any of the links in this pathway may lead to excessive cell proliferation and disease. A previous study has demonstrated that mutation of the gene encoding ALK-1 is associated with PAH (6). Jerkic *et al* (26), demonstrated that adult ALK-1 heterozygous mice demonstrated clear signs of PAH at an age of 9 weeks, including increased right ventricular systolic pressure, accompanied by elevated RVHI and the muscularization of surrounding arteries. Additionally, greater degrees of vascular occlusion and PVR appeared at an age of 36 weeks, indicating a worsening of the disease. These adult mice exhibited elevated levels of reactive oxygen species in their lungs, which may promote the development of PAH (26). In another study, Eyries *et al* (27), observed that a mutation of *ACVRL1* (the gene encoding ALK-1) exists in early carriers; PAH is observed in these patients, who exhibit a poor prognosis.

In the present study, 6 weeks following the subcutaneous injection of 60 mg/kg MCT, the mRVP, mPAP, RVHI, WT% and WA% in group M were significantly increased compared with those in the control. Furthermore, H&E staining revealed that the thickening of the pulmonary artery wall, narrowing of the lumen, and endothelial cell degeneration, swelling, shedding and necrosis had occurred, and morphological changes such as the infiltration of inflammatory cells around blood vessels were also observed. This suggested that MCT successfully induced the rat PAH model (28). In group M, with the increased pulmonary artery pressure and PVR, the protein expression levels of ALK-1 and Smad1 in the pulmonary artery were also significantly increased compared with those in the control group, indicating that the ALK-1 signaling pathway in group M was abnormal and suggesting that this signaling pathway may be involved in the occurrence of PAH and PVR. Therefore, it was speculated that ALK-1 upregulation caused increased ligand binding and complexation of Smad, and the resultant imbalance of the ALK-1/TGF-β/ALK-5 signaling pathway led to the activation of endothelial cells, decreased apoptosis, the proliferation of pulmonary arteries, and occlusion and reconstruction of the pulmonary arteries, thus causing PAH. However, this hypothesis requires further investigation.

As a precursor of reductive glutathione, NAC contains an active thiol group, is a commonly used expectorant drug and is able to dissolve mucus, and attenuate oxidation or inflammation. In addition, NAC is able to treat pulmonary fibrosis by inhibiting the proliferation of endothelial cells, reducing the TGF-β-induced generation of collagens and regulating gene

expression and signal transduction systems, thus exhibiting important roles for the reversal of PVR (29). In the present study, following treatment with NAC, the rats exhibited reductions in mRVP, mPAP, RVHI and PVR compared with the model rats. Furthermore, the protein expression levels of ALK-1 and Smad1 were reduced following treatment with NAC. It may be speculated that NAC downregulated the protein expression levels of ALK-1 and Smad1 in the ALK-1 signaling pathway, thus inhibiting the proliferation and migration of endothelial cells, improving PVR and reducing the pulmonary artery pressure. However, treatment with NAC did not completely reverse the pathological process, which indicates that other mechanisms are involved in the formation of PAH. Therefore, further research is required to investigate this.

The present study observed only the protein expression of ALK-1 and Smad1 in the pulmonary artery, and other cytokines in the ALK-1/TGF-β/ALK-5 signaling pathway were not studied. Therefore, the association of the ALK-1/TGF-β/ALK-5 signaling pathway with PAH requires further investigation. Furthermore, the mechanisms underlying the actions of NAC and the feasibility of using NAC in the treatment PAH have many uncertainties, and require further exploration. However, the present study indicates that ALK-1 and Smad1 are involved in the pathogenesis of PAH and PVR, and that NAC significantly inhibits the protein expression of ALK-1 and Smad1 in the lung tissue and pulmonary arteries in a rat model of PAH, thus attenuating PAH. In summary, the present study provides evidence for the future application of NAC in the treatment of PAH, and suggests its potential as a novel method for the treatment of PAH.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WY and XS conceived and designed the study. CL and WJ performed the experiments. XS and CL were major contributors in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Qingdao University (Qingdao, China). Ethical approval was obtained from the Ethics Committee of Qingdao University Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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