

Original Research Article

Notoginsenoside R1 improves monocrotaline-induced pulmonary arterial hypertension via modulation NF- κ B signaling in rats

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Abstract

Purpose: To investigate the potentials of notoginsenoside R1 (NGR1) in ameliorating inflammation and pulmonary vascular remodeling in rats with pulmonary arterial hypertension (PAH) induced by monocrotaline (MCT), and to examine the mechanisms underlying such effects.

Methods: Eight-week-old male Sprague Dawley rats were randomly divided into groups: control, MCT, MCT+5mg/kg NGR1, MCT+12.5mg/kg NGR1, and MCT + 25 mg/kg NGR1. Right cardiac catheterization was used to measure pulmonary hemodynamics. Pulmonary morphology was evaluated with the aid of H & E staining. Serum levels of inflammatory cytokines were measured using ELISA, while levels of inflammation-associated factors in the lung were measured using RT-PCR. NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) and I κ B α (nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha) protein levels were determined by western blot.

Results: Pulmonary hemodynamics and pulmonary morphology worsened following MCT injection and were accompanied by NF- κ B pathway activation and elevated levels of inflammation-associated factors. In contrast, MCT treatment followed by NGR1 treatment ameliorated MCT-induced PAH by improving pulmonary hemodynamics and pulmonary vascular remodeling while reducing NF- κ B activation and levels of inflammation-associated factors.

Conclusion: NGR1 exerts ameliorative effects on MCT-induced PAH by inhibiting NF- κ B pathway. Therefore, NGR1 may be a new potential therapy for PAH.

Keywords: Notoginsenoside R1, Monocrotaline-induced pulmonary arterial hypertension, Vascular inflammation, NF- κ B pathway

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INTRODUCTION

Pulmonary arterial hypertension (PAH), a multifactorial and life-threatening illness, presents as a persistent increase in pulmonary artery pressure and ultimately results in right

ventricular failure and death [1]. The pathogenesis of PAH includes pulmonary vasoconstriction, thrombosis, inflammation, and oxidative stress [2-4]. Accumulating evidence reveal pulmonary vascular inflammation has an essential role in PAH [3,5]. Inflammatory

responses promote abnormal contraction of pulmonary vessels and proliferation of pulmonary arterial smooth muscle cells (PASMCs), resulting in pulmonary arterial remodeling and pulmonary hypertension [6,7].

Nuclear factor-kappa B (NF- κ B) is closely associated with inflammation and cell proliferation [8]. Nuclear Factor- κ B increases the expression of tumor necrosis factor (TNF- α), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1), all of which are involved in the development of pulmonary hypertension [9,10]. Nuclear Factor- κ B activation is also a pathological characteristic of PASMC in patients with idiopathic PAH, and inhibition of NF- κ B activity is an effective therapy for PAH [11-13].

Classically, PAH is treated with calcium channel blockers, but current therapeutic strategies are focused on targets such as phosphodiesterase type 5 inhibitors, endothelin receptor antagonists, and prostanoids [14]. Although these medications provide clinical benefits, PAH patients continue to suffer from poor prognoses and low survival rates [15]. Therefore, it is urgent to develop novel therapeutic strategies for PAH treatment. *Panax notoginseng* saponins (PNS) are the major active ingredients derived from *Panax notoginseng*, widely used in traditional Chinese medicine to stanch bleeding [16]. Notoginsenoside R1 (NGR1) is a PNS with anti-inflammatory, cardioprotective, and anti-oxidative properties [17-19]. It alleviates inflammatory responses in human epithelial cells and inhibits vascular smooth muscle cell proliferation [17,20]. Therefore, the present study was designed to investigate whether NGR1 had a therapeutic effect on MCT-induced PAH in rats and to discover the mechanisms underlying any such effects.

EXPERIMENTAL

Establishment of PAH model

Eight-week-old male Sprague-Dawley rats were acquired from Shanghai SLAC Laboratory Animal Co., Ltd. All rats were maintained under standard conditions with constant temperature (22 ± 2 °C), constant humidity (60 ± 5 %), and a 12 h light/dark cycle. All animal experiments were carried out in accordance with the guidelines of International Ethical Guidelines for Biomedical Research [21], and were approved by the Ethics Committee of Huazhong University of Science and Technology (no. EC2017HU0048).

Rats were randomly divided into five treatment groups (n = 8 per group): control, MCT, MCT + 5 mg/kg NGR1, MCT + 12.5 mg/kg NGR1 and MCT + 25 mg/kg NGR1. A single dose of MCT (50 mg/kg; Sigma-Aldrich) was injected intraperitoneally (IP) to induce experimental PAH. Sham-treated animals were injected with an equivalent volume of saline. Subsequently, rats in the MCT + NGR1 groups were administered with NGR1 (Shanghai Tauto Biotech Co., Ltd.) by oral gavage daily for 21 days. Rats in control group and MCT-only treated control group were administered with equal volume of normal saline.

Hemodynamic measurements

Rats were anesthetized with 10 % chloral hydrate (IP injection; 3 ml/kg; Sigma-Aldrich) 21 days after MCT injection. A polyethylene catheter equipped with a pressure sensor was inserted into the jugular vein and directed through the right atrium and right ventricle into the pulmonary artery. Various parameters for assessing pulmonary artery pressure were then recorded. Finally, heart tissues of rats were excised and dissected into three parts: left ventricle (LV), interventricular septum (S), and right ventricle (RV). Right ventricular hypertrophy index (RVHI) was assessed as as in Eq 1.

$$RVHI = \text{weight}_{RV} / (\text{weight}_{LV} + \text{weight}_S) \dots\dots (1)$$

Hematoxylin and eosin (H & E) staining

The right lower lung lobe was harvested and fixed in 4 % paraformaldehyde (Sigma-Aldrich). The lung was embedded in paraffin and sectioned into tissue slices (5 μ m). H & E staining was carried out at room temperature with hematoxylin (0.5%; ZSGB-BIO) staining for 3 min, followed by eosin (0.5 %; ZSGB-BIO) staining for 2 min. Subsequently, the tissue slices were dehydrated and sealed. Finally, a light microscope (Olympus) was used to observe and photograph the H & E-stained slides.

Evaluation of serum cytokines

Rat plasma was harvested by retro-orbital injection, and the serum was separated by centrifugation (6000 rpm, 10 min) before collection. Levels of serum cytokines IL-6 and TNF α were measured by ELISA (R & D systems).

Determination of tissue RNA

RNA was isolated from exercised rat lung tissues which were homogenized in TRIzol reagent

(Invitrogen) with TissueLyser-192 (Shanghai JingXin). Message RNA (mRNA) was further purified using RNeasy Plus Micro and Mini Kits (QIAGEN). Complementary DNA (cDNA) was synthesized using the PrimeScript™ RT Reagent Kit (Takara), and quantitative RT-PCR was performed on an ABI 7900 instrument (Applied Biosystems). The primer sequences used to amplify *Icam1* (intercellular adhesion molecule 1), *Hmgb1* (high mobility group box 1), and *Actb* (actin) are listed in Table 1.

Western blotting

Harvested rat lung tissues were homogenized in sterile physiological saline and total protein extracts were prepared using RIPA lysis buffer (Beyotime). Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Equivalent amounts of protein were loaded into each lane of a 10 % polyacrylamide gel containing sodium dodecyl sulfate (SDS). Proteins were separated by electrophoresis and transferred to PVDF membranes (Millipore). Membranes were blocked in 5 % skim milk at room temperature for 2 h before adding primary antibodies against IκBα (Abcam), NF-κB p65 (Abcam) and β-actin (Abcam).

The membranes and antibodies were incubated at 4°C overnight. Membranes were washed three times in Tris-buffered saline containing 0.1 % Tween 20 before incubating with HRP-conjugated secondary antibodies (Abcam) for 1 h. Proteins were visualized with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) and analyzed using an ImageQuant LAS 4000 Mini (GE Healthcare Life Sciences).

Statistical analysis

Data were derived from three independent experiments and presented as the mean ± SEM. One-way analysis of variance (ANOVA) was used to evaluate the statistical differences among groups and $p < 0.05$ was considered statistical significant. SPSS and Prism softwares were used for data analyses.

Table 1: Primer sequences for *Icam1*, *Hmgb1* and *Actin*

Gene	Upstream primer	Downstream primer
<i>Icam1</i>	5'-TTCCCTTCTCTATTACCCC-3'	5'-GTGAGCGTCCATATTTAG-3'
<i>Hmgb1</i>	5'-GAGATCCTAAGAAGCCGAGA-3'	5'-CTTCCTCATCCTCTTCATCC-3'
<i>Actin</i>	5'-ATTGTCACCACTTTCTACAATGAGCTGCG-3'	5'-GCAGATGTGGATCAGCAAGCAGGAGTACGATG-3'

RESULTS

NGR1 improved pulmonary hemodynamics in rats with MCT-induced PAH

To determine whether notoginsenoside R1 (NGR1) might be an effective treatment for pulmonary arterial hypertension (PAH), this study used monocrotaline (MCT) to induce PAH in Sprague-Dawley rats. After an initial treatment with MCT (50 mg/kg), rats were treated with NGR1 (at concentrations of 0, 5, 12.5, or 25 mg/kg), once a day, for 21 days and then assessed. Hemodynamic parameters, including right ventricular systolic pressure (RVSP), mean pulmonary arterial pressure (mPAP), pulmonary arterial systolic pressure (PASP) and right ventricular hypertrophy index (RVHI), were significantly increased in the MCT-treated group (Figure 1). Notoginsenoside R1 (NGR1)-treatment restored normal hemodynamic parameters in MCT-treated rats in a dose-dependent manner. Importantly, in the MCT + 25 mg/kg NGR1 group, the levels of RVSP, mPAP, PASP, and RVHI were similar to their levels in the sham-treated control group (Figure 1). These results indicate that NGR1 may protect rats from MCT-induced PAH.

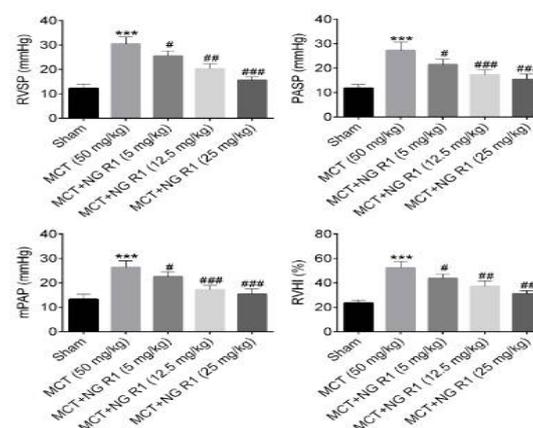


Figure 1: Effect of NGR1 treatment on pulmonary hemodynamics in MCT-induced PAH. RVSP, PASP, mPAP and RVHI were measured for each group 21 days after initial treatment with MCT (n = 8). *** $p < 0.001$ vs. sham group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. MCT group

Effect of NGR1 on MCT-induced pulmonary morphological changes

To assess whether NGR1 might protect against MCT-induced pulmonary artery remodeling, H&E staining of lung tissue sections was performed. Compared with the sham-treated group, the pulmonary arteries of the MCT-treated group had thicker walls and narrower lumens (Figure 2). In addition, immune cell infiltration was evident, indicating the presence of pulmonary interstitial inflammation (Figure 2). In contrast, NGR1 treatment led to a more normal pulmonary vascular wall structure and less inflammatory infiltration, again in a dose-dependent manner. Strikingly, very little pulmonary artery remodeling was detected in the MCT + 25 mg/kg NGR1 group (Figure 2). These results suggest that NGR1 ameliorates MCT-induced pulmonary arterial remodeling.

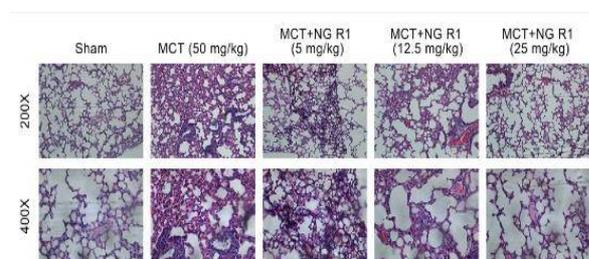


Figure 2: Protective effects of NGR1 on pulmonary morphological changes induced by MCT. H & E staining of rat lung tissues in different groups (n = 6 - 8)

NGR1 inhibited MCT-induced pulmonary vascular inflammation

To investigate the mechanisms underlying the influence of NGR1 on MCT-induced PAH, serum protein levels of the proinflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) were determined. Tumor Necrosis Factor- α and IL-6 levels significantly ($p < 0.001$) increased in rats treated with MCT alone, but when MCT treatment was followed by NGR1 treatment, a dose-dependent reductions in IL-6 and TNF- α levels was observed (Figure 3 A). Similarly, *Icam1* (intracellular adhesion molecule 1) and *Hmgb1* (high mobility group box 1) expressions were elevated in lung tissue from the MCT-treated group when compared to the sham-treated group, but MCT treatment followed by NGR1 treatment restored normal expression levels (Figure 3 B). These results suggest that NGR1 reduces pulmonary inflammatory response in rats with MCT-induced PAH.

NF- κ B signaling pathway mediates the protective effects of NGR1 in MCT-induced PAH rats

The inflammatory response depends, in part, on the activation of NF- κ B and the degradation of I κ B α . Therefore, to investigate how NGR1 might inhibit the expression of pro-inflammatory factors like TNF- α and IL-6, NF- κ B and I κ B α protein levels were determined by western blot analysis. Compared to the sham-treated group, rats treated with MCT had significantly ($p < 0.001$) increased NF- κ B levels and decreased I κ B α levels (Figure 3 C). Conversely, in the MCT + NGR1 groups, NF- κ B levels decreased, whereas I κ B α levels increased significantly ($p < 0.001$) (Figure 3 C). These results demonstrate that the NF- κ B signaling pathway is crucial for the protective effects of NGR1.

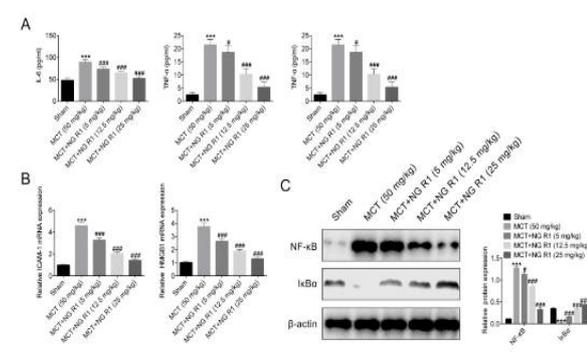


Figure 3: Effect of NGR1 treatment on the expressions of MCT-induced markers in pulmonary vascular inflammation. (A) Levels of IL-6 and TNF α in rat serum determined by ELISA. (B) Expressions of *Icam1* and *Hmgb1* in rat lung tissue determined by qRT-PCR. (C) Protein levels of NF- κ B and I κ B α in rat lung tissue. *** $p < 0.001$ vs. sham group. # $p < 0.05$, ### $p < 0.001$ vs. MCT group

DISCUSSION

In the current study, rats with MCT-induced PAH exhibited increases in pulmonary hemodynamics, pathological changes in pulmonary structure, and pulmonary vascular inflammation. In rats treated with MCT followed by treatment with NGR1 for 21 days, these monocrotaline-induced effects were attenuated. Mechanistically, the protective effect of NGR1 on MCT-induced PAH depends on the suppression of NF- κ B signaling pathway. MCT, a two-pyrrole alkaloid, is transformed into MCT pyrrole (MCTP) in the liver by P450 monooxygenase [22]. MCT pyrrole is harmful to pulmonary arterial endothelial cells leading to vascular injury, remodeling, and inflammation [22]. The pathological symptoms induced by MCT in rats are comparable to the symptoms of human PAH, making the MCT-induced PAH rat model suitable for PAH studies [22]. In the

present study, MCT treatment significantly increased RVSP, PASP, mPAP, and RVHI; thickened the pulmonary artery wall and narrowed its lumen; and enhanced inflammatory infiltration, thus validating the MCT-induced PAH rat model. These MCT-induced effects were alleviated by NGR1 administration.

High levels of the pro-inflammatory cytokines TNF- α and IL-6 might induce pulmonary artery endothelial cell damage and abnormal PASMC proliferation, and contribute to PAH [3,7]. ICAM-1, an intercellular adhesion molecule present in endothelial cells, facilitates leukocyte endothelial transmigration [23]. High Mobility Group Box-1 Protein, produced by activated immune cells, acts as a cytokine mediator of inflammation [24]. Therefore, ICAM-1 and HMGB1 can be regarded as biomarkers of inflammation. In this study, NGR1 reduced the elevated levels of IL-6, TNF- α , ICAM-1 and HMGB1 induced by MCT, thereby demonstrating that NGR1 has possible anti-inflammatory effects.

NF- κ B, a major transcription factor, regulates many genes involved in both the innate and adaptive immune responses [25]. Normally, dimers of NF- κ B bind to inhibitory I κ B proteins in the cytoplasm. Upon stimulation, I κ B kinase phosphorylates the I κ Bs, thereby targeting them for degradation and freeing NF- κ B to translocate into the nucleus to induce inflammatory cascades [26]. In the current study, the MCT-induced increase in NF- κ B protein levels and decrease in I κ B α levels were inhibited by NGR1. These findings indicate that suppression of the NF- κ B signaling pathway contributes to the molecular mechanism underlying the anti-inflammatory effects of NGR1.

CONCLUSION

The findings of the present study demonstrate that NGR1 exerts effects that counter MCT-induced PAH, and these effects are associated with NF- κ B pathway. Thus, NRG1 is a potential novel therapy for PAH.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities

pertaining to claims relating to the content of this article will be borne by the authors. Shanglong Yao and J Yinglu Feng designed all the experiments and revised the manuscript. Na Hu and Min Tang performed the experiment.

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