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Celastrol attenuates the remodeling of pulmonary vascular and right ventricular in monocrotaline-induced pulmonary arterial hypertension in rat

Dr. Huayang Li
The First Affiliated Hospital of Sun Yat-Sen University, Zhongshan II Rd 58, 510080 Guangzhou, China

Summary

Pulmonary arterial hypertension is a progressive angio-proliferative disease associated with high morbidity and mortality rates. Although the histopathology of pulmonary arterial hypertension is well described, its therapeutic option remains unsatisfactory.It is increasing being recognized that early and persistent inflammation plays an indispensable role in the development and progression of PAH.

Celastrol, isolated from the root extracts of *Tripterygium wilfordii* (thunder god vine), is a quinone methide triterpene. Increasing evidence suggests that celastrol exerts powerful effects on anti-inflammatory and antioxidant activities.

This study investigated the effect of celastrol treatment on right ventricular dysfunction, remodeling, and pulmonary vascular remodeling in pulmonary arterial hypertension rats as well as its possible mechanisms.

Registration details	
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1. General Information

Keywords

Pulmonary arterial hypertension, Celastrol, Pulmonary vascular remodeling, Right ventricular,

Funding sources

The National Natural Science Foundation of China (grant nos. 81770319 and 81900294)

International code of classification

BB01.0 - Pulmonary arterial hypertension

Additional remarks

right ventricular remodeling and dysfunction

2. Study design

Introduction

Pulmonary arterial hypertension is a progressive angio-proliferative disease associated with high morbidity and mortality rates. Although the histopathology of pulmonary arterial hypertension is well described, its therapeutic option remains unsatisfactory.It is increasing being recognized that early and persistent inflammation plays an indispensable role in the development and progression of PAH.

Celastrol, isolated from the root extracts of *Tripterygium wilfordii* (thunder god vine), is a quinone methide triterpene. Increasing evidence suggests that celastrol exerts powerful effects on anti-inflammatory and antioxidant activities.

Type of research

Confirmatory

Hypothesis of your study

Celastrol improves right ventricular function, attenuates right ventricular and pulmonary vascular remodeling in rats with pulmonary arterial hypertension.

Study design

The rats studied 4 weeks after a single subcutaneous injection of MCT (60 mg/kg; Sigma, St. Louis, MO, USA) or equal volume of saline (the Normal group). From the first day, each rat was tagged with an ear tag to identify it, and MCT-injected rats were randomly divided into two groups. The rats from one group received daily intraperitoneal injection of celastrol (1 mg/kg/day, TargetMol, Shanghai, China) for 4 weeks, and an equal volume of vehicle [0.9% dimethylsulfoxide+2% Tween 80+97.1% saline] was administered to another group. Therefore, there were three groups and 35 rats: the normal group (n=10), the vehicle group (n=13), the celastrol group (n=12). The grouping would not be announced until all data had been collected.

Method of blinding

One day after injection of MCT or equal volume of saline, each rat was tagged with an ear tag to identify it and MCT-injected rats were randomly divided into the vehicle group and the celastrol group. Then the number of the ear tag will be recorded in the EXCEL table to clarify the grouping information and only Huayang Li had the table. The grouping would not be announced until all data had been

collected, which meant that no one knew which rat belonged to which group until the data had been collected.

Method of randomization

To minimize potential confounders, the injection sequence of the three groups was different every day and the order of measurements was also random.

3. Methods

3. 1. Echocardiography

Description of the method

The rats were anaesthetized with continuous isoflurane inhalation (1.5-3%) and maintained with spontaneous respiration. Transthoracic echocardiography was performed by a 25 MHz linear array transducer (Vevo 2100, VisualSonics, Toronto, Canada). Short axis M-mode recordings were obtained to measure left ventricle ejection fraction (LVEF). Pulsed-wave Doppler recordings of the right ventricular overflow tract were used to measure pulmonary acceleration time (PAT), peak ejection time (PET) and pulmonary artery velocity time integral (PA-VTI). Tricuspid annular plane systolic excursion (TAPSE) was measured by using M-mode across the tricuspid valve annulus at the RV free wall.

Narcotic/analgesic treatment

isoflurane inhalation (1.5-3%)

Drugs/substances

Not applicable

Antibodies

Not applicable

Cell lines, viruses, DNA or RNA constructs and bacteria

Not applicable

3. 2. RV catheterization and hypertrophy index

Description of the method

After echocardiography, terminal invasive haemodynamic measurements were performed to confirm RV pressure via RV catheterization. The rats were anaesthetized with pentobarbital sodium (40 mg/kg) and fixed on plank. Then, the right jugular vein was isolated for intubation and lidocaine was use for local analgesia. The PE-50 tube was filled with heparin saline, connected to a pressure sensor (Techman, Chengdu, China) and inserted into the right external jugular vein. The appearance of the ventricular pressure wave indicated that the catheter reached the RV. Then the RV systolic pressure (RVSP) was analysed.

Narcotic/analgesic treatment

pentobarbital sodium (40 mg/kg)

Drugs/substances

Not applicable

Antibodies

Not applicable

Cell lines, viruses, DNA or RNA constructs and bacteria

Not applicable

3. 3. Masson staining and haematoxylin-eosin (H&E) staining

Description of the method

After haemodynamic measurements and blood withdrawals, the heart and lungs were excised and harvested for fibrosis, morphometric and histologic analysis. The heart and middle lobe of the right lung were dissected and fixed in 4% paraformaldehyde for 24 h, embedded in paraffin and sectioned. Heart sections were stained with Masson’s trichrome and lung sections were stained with H&E. A light microscope (Carl Zeiss, Jena, Germany) was used for overall histological assessment. RV fibrosis was assessed by Image-Pro Plus software (Version 6.0, Media Cybernetics, Silver Springs, MD, USA). Pulmonary arterial medial wall thickness (WT) was calculated by the following formula: WT (%) = $area_{ext}$ - $area_{int}$ / $area_{ext}$ × 100, where $area_{ext}$ and $area_{int}$ are areas bounded by external and internal elastic lamina, respectively

Narcotic/analgesic treatment

Not applicable

Drugs/substances

Not applicable

Antibodies

Not applicable

Cell lines, viruses, DNA or RNA constructs and bacteria

Not applicable

3. 4. Immunohistochemistry

Description of the method

The expression of α-smooth muscle actin (a-SMA) and CD68 in the middle lobe of the right lung was detected by immunohistochemistry staining. The tissue slides were deparaffinized with xylene and then serially rehydrated with ethanol. Following brief proteolytic digestion and peroxidase blocking, the slides were incubated with primary antibodies against α-SMA (19245S, Cell Signaling Technology, USA, at a dilution of 1:500) and CD68 (GB11067,

Servicebio, Wuhan, China, at a dilution of 1: 500) at 4# overnight. The slides were first washed to remove the unbound primary antibodies and then incubated with a peroxidase conjugated secondary antibody. The specifically bound secondary antibody was detected with a DAKO EnVision detection System (Dako, Glostrup, Denmark).

Narcotic/analgesic treatment

Not applicable

Drugs/substances

Not applicable

Antibodies

α-SMA# CD68

Cell lines, viruses, DNA or RNA constructs and bacteria

Not applicable

3. 5. Western blot

Description of the method

Flash-frozen inferior lobes of the right lungs were homogenized in radioimmunoprecipitation assay buffer supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Protein content was determined by using a BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Equivalent amounts of protein were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) (10%) and transferred to polyvinylidene difluoride membranes. After being blocked for 1 h in 5% nonfat dry milk and Tris-buffered saline, the membrane was incubated with the desired primary antibody for 8 h at 4#. The membrane was then treated with appropriate horseradish peroxidase conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA), and the immunoreactive bands were detected by chemiluminescence (ECL) reagents (Merck Millipore, Billerica, MA, USA). Specific antibodies against TGF-β1(sc-130348, Santa Cruz, CA, USA), IkBα (4814S, Cell Signaling Technology, Danvers, MA, USA), phosphorylated IKKα/β (2697S, Cell Signaling Technology, Danvers, MA, USA), p65(sc-8008, Santa Cruz, CA, USA), and GAPDH (60004-1-lg, Proteintech, Chicago, IL, USA) were used in the immunoblot assay. For analysis, the expression of target proteins was normalized to GAPDH.

Narcotic/analgesic treatment

Not applicable

Drugs/substances

Not applicable

Antibodies

TGF-β1#IkBα#phosphorylated IKKα/β#p65#GAPDH

Cell lines, viruses, DNA or RNA constructs and bacteria

Not applicable

3. 6. Reverse Transcription-quantitative Polymerase chain Reaction

Description of the method

The superior lobes of the right lungs were isolated from the rats and homogenized, and total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. One thousand nanograms of RNA was reverse-transcribed to cDNA by using the PrimeScript™ RT reagent Kit (RR037A, Takara, Tokyo, Japan). The PCR primers were designed and synthesized by Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). The following specific primers were generated and used: for Collagen I, forward primer: GTACATCAGCCCAAACCCCA, reverse primer: TCGCTTCCATACTCGAACTGG; for Collagen III, forward primer: TGGTGGCTTTCAGTTCAGCTA, reverse primer: ATTGCCATTGGCCTGATCCA; for TGF-β1, forward primer: CTGCTGACCCCCACTGATAC, reverse primer: AGCCCTGTATTCCGTCTCCT; for monocyte chemotactic protein 1 (MCP-1), forward primer: TGTCTCAGCCAGATGCAGTT, reverse primer: CAGCCGACTCATTGGGATCA; for IL-1β, forward primer: TAGCAGCTTTCGACAGTGAGG, reverse primer: CTCCACGGGCAAGACATAGG; for IL-6, forward primer: CATTCTGTCTCGAGCCCACC, reverse primer: GCTGGAAGTCTCTTGCGGAG; for IL-10, forward primer: CCTGGTAGAAGTGATGCCCC, reverse primer; GACACCTTTGTCTTGGAGCTTAT; for GAPDH, forward primer: CGCTAACATCAAATGGGGTG, reverse primer: CGCTAACATCAAATGGGGTG. Quantitative PCR was performed using TB Green® Premix Ex Taq™ II (RR820A, Takara, Tokyo, Japan), and the samples were assayed in duplicate according to the manufacturer's instructions in a BioRad CFX96 PCR system (Bio-Rad, Hercules, CA, USA). For analysis, the expression of target genes was normalized to GAPDH.

Narcotic/analgesic treatment

Not applicable

Drugs/substances

Not applicable

Antibodies

Not applicable

Cell lines, viruses, DNA or RNA constructs and bacteria

Not applicable

4. Statistics

4. 1. one-way ANOVA

Assigned method(s)

Echocardiography

RV catheterization and hypertrophy index
Masson staining and haematoxylin-eosin (H&E) staining
Immunohistochemistry
Western blot
Reverse Transcription-quantitative Polymerase chain Reaction

Main endpoints

RV systolic pressure

Secondary endpoints

Not provided

Sample size calculation

Monocrotaline rat pulmonary atrial hypertension (PAH) model is a widely used experimental PAH model. It has high stability and can efficiently reproduce the pathophysiology of clinical PAH (1). After reading the studies on rat PAH induced by monocrotaline, we found that most studies used 5-11 rats per group (2-4). Considering that the rats may die from PAH during the observation period, and in order to improve the power of test, we finally used the current sample size: the normal group (n=10), the vehicle group (n=13), the celastrol group (n=12).

Primary statistical analysis

one-way ANOVA

Exclusion criteria

1.Unexplained severe weight loss#2. Died from a fight#3.severe skin disease#4. Obivious penumonia

5. Animals

5. 1. Rats (Rattus norvegicus)

Animal strain/breed

Sprague-Dawley,purchased from Laboratory Animal Center of Sun Yat-sen University, Guangzhou, China

Genetically modified

No

Sex

Male

Further characteristics of the animals (e.g. age, body weight, size)

6-8weeks#220-250g

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Housing conditions

Rats housed in a specific pathogen free room with a 12 h light/dark cycle and stable humidity and room temperature (40~70% and 20 ~ 25#, respectively). Water and rat chow were provided ad libitum.3 or 4 rats were housed in each cage and cage changes per 3 days.

Refinement

If the rats has the following conditions:1.Unexplained severe weight loss#2.severe skin disease#3. Obivious penumonia, it is euthanized by anesthesia.