Rebaudioside B attenuates lung ischemia-reperfusion injury associated apoptosis and inflammation

Jian Huang  
Lanzhou University Second Hospital, Lanzhou University

Jianbao Yang  
Lanzhou University Second Hospital, Lanzhou University

Rongzhi Zhang  
Lanzhou University Second Hospital, Lanzhou University

Shixiong Wang  
Lanzhou University Second Hospital, Lanzhou University

Xingdong Cheng  
Lanzhou University Second Hospital, Lanzhou University

Jian Li  
Lanzhou University Second Hospital, Lanzhou University

Yongnan Li  
Lanzhou University Second Hospital, Lanzhou University

Bingren Gao (gaody16@lzu.edu.cn)  
Lanzhou University Second Hospital, Lanzhou University

Research Article

Keywords: Rebaudioside B, lung ischemia-reperfusion injury, apoptosis, A549 cells, high-throughput screening, oxygen-glucose deprivation/recovery

Posted Date: June 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1700666/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Currently no proven effective treatment exists for lung ischemia-reperfusion injury (LIRI). Natural compounds represent a source of new drugs for the treatment of different diseases. The present study aims to investigate Rebaudioside B (Reb B) as a potential compound to the treatment of LIRI in vivo and in vitro. The present study includes the human pulmonary alveolar cells line with epithelial type II A549 under oxygen-glucose deprivation/recovery (OGD/R) to high-throughput in vitro cell viability screening to identify the most promising compound tested candidates. Moreover, an in vivo rat model of lung ischemia-reperfusion was used to assess the potential protective effect of Reb B. Reb B was identified as the most promising compound among the natural compounds tested, being identified by high-throughput screening. Reb B enhances cell viability by reducing apoptosis in A549 OGD/R models. In the in vivo model of LIRI, preconditioning with Reb B significantly reduced apoptotic cells, perivascular edema, and neutrophil infiltration in lung tissues. In addition, Reb B inhibits LIRI lung inflammation mainly by increasing IL-10 levels, while decreasing IL-1β and IL-8 levels. The overall results suggest that Reb B has a promising protective effect in LIRI caused by the inhibition of the mitochondrial apoptotic pathway and by decreasing the inflammatory response.

Introduction

Lung ischemia-reperfusion injury (LIRI) is a complex pathophysiological process that occurs because of different clinical conditions, including cardiac arrest, trauma, pulmonary thrombosis, lung transplantation and cardiopulmonary bypass [1]. Pulmonary ischemia-reperfusion injury corresponds to the damage of the pulmonary vascular endothelium and alveolar epithelium [2]. LIRI represents the main cause of early primary graft dysfunction and failure after lung transplantation [3]. In this context, natural compounds may represent a promising source of new compounds to treat LIRI. Natural products represent an extensive reservoir of diverse chemical compounds with novel biological targets and mechanisms of action [4]. In the present study, a high-throughput screen of a compound library composed of 2,661 single compounds purified from natural products was tested to identify the most effective compound on an in vitro model of ischemia-reperfusion injury, using A549 cells, i.e., a human pulmonary alveolar cells line with epithelial type II cell properties. The results identified Rebaudioside B (Reb B) as the most promising compound. Reb B is a minor component of steviol glycosides (SGs) that can be founded mainly in stevia leaf [5]. The SGs despite being 300 times sweeter than sucrose are non-caloric and therefore do not increase blood sugar levels [6]. For the above-mentioned reasons, SGs are widely used as a nontoxic food additive for sweeteners [7]. In this context, Reb B was selected in this study because it showed a high inhibition of apoptosis despite the mechanism of inhibition of apoptosis in LIRI was not described to the best of our knowledge in the literature. The present study aims to investigate the potential protective effect of Reb B in an in vivo model of LIRI.

Materials And Methods
The present study comprised the *in vitro* (Experiment 1) and the *in vivo* (Experiment 2) studies to assess the efficacy of Reb B in LIRI. Experiment 1 was designed to screen the most promising compound from natural products libraries (2,661 compounds) that could inhibit ischemia-reperfusion injury of A549 cells. The effects of Reb B on lung function and inflammation after ischemia-reperfusion injury in rats were conducted in Experiment 2. This protocol was approved by the institutional animal experimental ethics committee of Lanzhou University Second Hospital (Lanzhou, Gansu, China) (No. P2021-019).

**Experiment 1**

**Establishment of A549 cells OGD/R (oxygen-glucose deprivation/recovery) model**

A549 cells were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Solarbio, Beijing, China) supplemented with 10% fetal calf serum (FBS) (GIBCO, USA), 100 U/ml penicillin, and 200 µg/ml streptomycin (GIBCO, USA) and incubated in the humidified incubator (Thermo Fisher, Waltham, USA), containing 5% CO2 at 37 °C. The culture medium was changed once every 2 days. To simulate LIRI *in vitro*, A549 cells were subjected to OGD/R. To induce OGD/R injury, cells were treated with sugar-free and serum-free DMEM medium and incubated in a tri-gas incubator (37 °C, 1% O2, 5% CO2) (Thermo Fisher, Waltham, USA), after OGD for 12h, the cells were changed with fresh culture medium and were returned to normal culture conditions.

**Compounds candidates identified through high-throughput screening and evaluate the effect on A549 cells OGD/R model**

A549 cells were seeded on 96-well plates and allowed to attach for 24h. Before OGD/R, cells were previously treated with each of 2,661 natural compounds from a natural product library at a final concentration of 10 µM for 12h. After OGD/R treatment, the CCK8 cell proliferation kit (Biosharp, Hefei, China) was used according to the manufacturer's instructions. Quantitative analysis of cell viability allowed us to identify the most promising compound, namely Reb B. To study the cytotoxic effects of Reb B on A549 cells under OGD/R model, different concentrations were tested, namely 1 mM, 10 mM, 100 mM, and 1000 mM. To understand the role of the most promising candidates on A549 cells OGD/R model, three groups of cells were used, namely the vehicle group, the OGD/R group, and the OGD/R + compounds candidates group. The cells before OGD/R modeling were incubated with a concentration of 10 mM Reb B. The protective mechanism of Reb B was studied by Hoechst 33258 staining, Western Blot, and Reverse Transcription-Quantitative PCR (RT-qPCR).

**Experiment 2**

**Animals**

In this study, a total of 17 male Sprague Dawley rats (weighing 250 ± 25g; 8 weeks old) were used. All animals were housed in smooth-bottomed plastic cages at 22°C with a 12-hour light/dark cycle. All experiments were performed between 8:00 am and 6:00 pm during the light phase of the cycle.
Experimental LIRI in vivo model

The rats were randomly distributed into three groups: Sham group (n = 5), LIRI group (n = 6), and LIRI + Reb B group (n = 6). Reb B was intravenously injected 60 min before thoracotomy as a pretreatment. Rats were heparinized (300 U/kg ip.) and anesthetized (pentobarbital sodium, 50 mg/kg ip.). After anterior neck soft tissue dissection after sterilization to expose the trachea, a tracheotomy was performed, and artificial ventilation was started (tidal volume 8ml/kg, frequency 72/min, positive end-expiratory pressure of 2 cm H$_2$O, FiO$_2$ 100%). Reb B (5 mg/kg) dissolved with dimethylsulfoxide (DMSO) was administered through the femoral vein in the LIRI + Reb B group, and an equal volume of DMSO was administered in both Sham and LIRI group. The animals were placed in the left lateral decubitus position, and then a left thoracotomy was performed through the fourth or fifth intercostal space, the muscular layer and pleura were gently dissected to expose the heart and lung. In the LIRI groups treated with vehicle or Reb B (LIRI and LIRI + Reb B groups, respectively), the left pulmonary hilum was occluded for 90 minutes with a non-invasive vascular clamp to induce ischemia. During the left lung ischemia, tidal volume parameters drop to 5 ml/kg. After 90 minutes of ischemia, the non-invasive vascular clamp was removed to begin reperfusion for 120 minutes, and the tidal volume recovered to 8mL/kg. Rats in the Sham group underwent 210 minutes of perfusion without ischemia. After the experiments, rats were euthanized using overdoses pentobarbital administered intravenously. Subsequently, the lung tissues and serum were collected for the following examination. The experimental flow chart is shown in Fig. 3.

Chemicals and Antibodies

The natural product library used in this study was purchased from Selleck Chemicals (#L1400, Houston, USA). The library contains a collection of 2,661 natural compounds (purity ≥ 99%) supplied as solutions dissolved in DMSO and water. The primary antibodies used were anti-Bcl2 (Proteintech, Wuhan, China), anti-Bax (Proteintech, Wuhan, China), anti-Caspase3 (Proteintech, Wuhan, China), anti-Cleaved Caspase3 (Cell Signaling Technology, Danvers, MA, USA), anti-Myeloperoxidase (Cambridge, MA, USA).

Cell counting kit-8 assay

The viability of cells was measured using a cell counting kit-8 (CCK8, Biosharp, Hefei China) according to manufactures instructions. The absorbance (A) was measured at the wavelength of 450 nm by a microplate reader (Thermo Fisher, Waltham, USA). In addition, the cell survival rate was calculated according to the following formula:

\[
\text{Cell viability (\%)} = \frac{(A_{\text{experiment}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100\%.
\]

Hoechst 33258 staining
Morphological analysis was performed using Hoechest 33258 staining, aiming to investigate cells apoptosis. After treatment, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde at room temperature for 15 min. After, cells were incubated with Hoechst 33258 at room temperature for 10 min, followed by observation under a fluorescence microscope (Olympus, Tokyo, Japan). Strong fluorescence can be observed in the nuclei of apoptotic cells, while a weak fluorescence was observed in non-apoptotic cells. The apoptotic rate was calculated by counting the number of apoptotic cells in 100 cells under a random field of view, being the formula used as followed: Apoptosis rate = number of apoptotic cells/total number of cells × 100%.

**Western Blot assay**

The total protein content extracted from cells and lower left lung tissues was extracted using Radioimmunoprecipitation assay buffer (RIPA buffer) lysis buffer (50 mM Tris (pH 7.4), 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 0.1% SDS, sodium orthovanadate) with protease inhibitor. Supernatants were extracted and determined by Bicinchoninic acid (BCA) assay for protein concentration. The proteins were transferred to 0.45 μm polyvinylidene fluoride (PVDF) membranes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were blocked with 5% defatted milk for 2 hours at room temperature and then incubated overnight at 4 °C with primary antibodies, β-actin (Proteintech, Wuhan, China) was used as a reference gene in this experiment. Finally, positive signals were developed by SuperSignal West Pico Plus ECL Kit (Thermo Fisher, Waltham, USA) and analyzed using ImageJ 1.49 (National Institutes of Health, USA).

**Reverse-transcription quantitative PCR (RT-qPCR) analysis**

The total content of RNA extracted from cells and lung tissues was isolated from each group using Trizol reagent (Takara, Kusatsu, Japan) and in agreement with the manufacturer’s protocol. The yield of RNA was determined using a Nano Drop spectrophotometer (Thermo Fisher, Waltham, USA), being 1000 ng of RNA used for the synthesis of complementary DNA and subsequent PCR amplification. Real-time PCR was performed using TB Green qPCR Mix Plus (Takara, Kusatsu, Japan) and the CFX96TM Real-time Detection System (Bio-Rad, CA, USA). The β-actin was used as an endogenous reference. Data was analyzed using the relative standard curve method according to the manufacturer’s protocol. All data was normalized against β-actin mRNA levels and expressed as fold increases relative to controls. The primer sequences of the tested genes were as follows: caspase3 Forward 5'-AGAGGGGATCGTTGTAGAAGTC-3' and Reverse 5'-ACAGTCCAGTTCTGTACCACG-3'; bcl2 Forward 5'-CCAGCGTATATCGGAATGTGG-3' and Reverse 5'-CCATGTGATACCTGCTGAGAAG-3'; bax Forward 5'-CCCGAGAGGTCTTTTTCCGAG-3' and Reverse 5'-CCAGCCCATGATGGTTCTGAT-3'; β-actin Forward 5'-TACCCTGCGCATTGCTGTA-3' and Reverse 5'-TCCTTCTGCATCCTGCGCAAT-3'.

**Histological Analysis**
The middle part of the left lung tissues samples was fixed in 4% paraformaldehyde, being embedded in paraffin and sectioned (5 mm) using Hematoxylin-Eosin (H&E) staining, TUNEL nuclear staining, and immunohistochemistry (IHC). Myeloperoxidase is an important indicator of neutrophil activation and is associated with the absolute number of neutrophils. To detect the neutrophil using IHC staining, the avidin-biotin complex method was used. After deparaffinization and blocking with 10% goat serum, the section was incubated with a primary antibody (anti–myeloperoxidase antibody) and then with a biotin-labeled secondary antibody in a standard manner. TUNEL staining was performed to detect cell apoptosis. The progression of perivascular edema was evaluated as described by comparing the perivascular area to the total vessel area. All the scores were repeated three times using a double-blind method. TUNEL staining (In Situ Death Cell Detection Kit, Roche, Belgium) with DAB staining was performed following the manufacturer’s instruction. The number of myeloperoxidase-positive cells and apoptotic cells was expressed by the average number in 5 randomly chosen high-power fields (HPFs) per section at a magnification of 400´. The perivascular cuff area was measured in 5 randomly chosen vessels per section at a magnification of 200´.

**Lung wet-to-dry weight ratio**

Calculate the lung wet-to-dry weight ratio using the upper part. Wet weights were measured shortly after harvest and dry weights were measured after drying at 100 °C for 24 hours. This ratio was calculated by dividing the wet weight by the dry weight.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Briefly, 10 mg of lower left lung tissues were homogenized in 600 ml of PBS with protease inhibitors (Roche, Belgium). The tissue lysate was centrifuged at 12500 rpm for 15 minutes, being the cleared supernatant collected and used for ELISA assay. The concentrations of TNF-a, IL-1b, IL-6, IL-8, and IL-10 in tissue lysates of the lung were measured using ELISA kits (Enzyme-linked Biotech, China) according to the manufacturer’s instructions.

**Statistical Analysis**

Data are presented as the mean ± standard error of the mean. Comparisons between 2 independent groups were performed using the Mann–Whitney U test. GraphPad Prism Software (Version 8; GraphPad Software Inc, La Jolla, Calif) was used for statistical analysis. All P-values were adjusted with Tukey post hoc test for multiple comparisons, with a P-value < 0.05 considered to be statistically significant.

**Results**

**Natural compound screening identifies Reb B as a specific anti-apoptosis molecule on A549 cells OGD/R model**

The “high-throughput screening” approach was used to screen existing compounds with the potential to protect A549 cells viability in a model of OGD/R. Fig. 1A depicted a schematic diagram of the study
protocol comprising the Natural Products Libraries used in this study with a total of 2,661 compounds used in the screening. The protection was measured as presented in percentage, representing the cell viability assay. With this experiment was then selected the most promising candidate compound for the following experiments, namely Reb B (Fig. 1B).

**Effect of Reb B on A549 cells OGD/R model cell viability**

CCK8 assay was used to detect the effect of Reb B used in different concentrations on A549 cells OGD/R cell viability. The results show that the viability of OGD/R-treated A549 cells are significantly lower when compared with the Vehicle group ($38.86 \pm 2.88$ vs $100.00 \pm 0.00$, $P < 0.001$, Fig. 2A). However, Reb B treatment markedly increased the cell viability after OGD/R injury. The cell viability of the OGD/R + Reb B group was the highest compared with that of the OGD/R group at a dose of 10 µM ($91.01 \pm 0.38$ vs $38.86 \pm 2.89$, $P < 0.001$, Fig. 2A). Therefore, 10 mM of Reb B was used for further experiments.

**Effect of Reb B on apoptosis of OGD/R in A549 cells**

The effect of Reb B on cell apoptosis was further analyzed using A549 cells OGD/R model. Hoechst33258 staining revealed the presence of many apoptotic cells after OGD/R treatment (Fig. 2B). The OGD/R + Reb B group showed a significantly lower apoptosis rate compared with the OGD/R group ($31.90 \pm 2.54$ vs $89.55 \pm 1.43$, $P < 0.001$, Fig. 2C). RT-qPCR analysis showed a significantly reduced $caspase3/b$-actin and $bax/b$-actin level in the OGD/R + Reb B group compared with the OGD/R group ($1.22 \pm 0.02$ vs $1.38 \pm 0.02$, $P < 0.001$ and $1.18 \pm 0.02$ vs $1.48 \pm 0.04$, $P < 0.001$ Figure 2D), but the level of $bcl2/b$-actin in OGD/R + Reb B group was higher than that in OGD/R group ($0.98 \pm 0.03$ vs $0.55 \pm 0.02$, $P < 0.001$, Fig. 2D). Similarly, WB analysis showed that the Caspase3/b-actin, Cleaved-Caspase3/b-actin and Bax/Bcl2 level in the OGD/R + Reb B significantly reduced compared with the OGD/R group ($0.76 \pm 0.02$ vs $1.03 \pm 0.02$, $P < 0.001$ and $0.61 \pm 0.05$ vs $1.02 \pm 0.06$, $P < 0.001$ and $0.84 \pm 0.04$ vs $1.35 \pm 0.05$, $P < 0.001$, respectively, Fig. 2F).

**Histological Effect of Reb B**

The perivascular cuff area was measured in 5 vessels per histologic section, which resulted in 25 vessels for the Sham group and 30 vessels for both the LIRI and LIRI + Reb B groups. The index of the perivascular cuff area was calculated to the vessel area to eliminate variations related to vessel size [8]. The index was significantly lower in the LIRI + Reb B group than in the LIRI group ($51.40 \pm 1.63$ vs $71.60 \pm 1.50$, $P < 0.001$, Fig. 4B) and the wet-to-dry weight ratio was significantly lower in the LIRI + Reb B group than in the LIRI group ($5.57 \pm 0.20$ vs $7.73 \pm 0.23$, $P < 0.001$, Fig. 4C). TUNEL staining showed that the number of apoptotic cells was lower in the LIRI + Reb B group than in the LIRI group ($24.80 \pm 2.58$ vs $41.00 \pm 1.05$, $P < 0.001$, Fig. 5C). The number of neutrophil infiltrations into the alveolar area was markedly reduced in the LIRI + Reb B group compared with the LIRI group ($13.20 \pm 1.32$ vs $44.40 \pm 2.09$, $P < 0.001$, Fig. 6B)

**Effects of Reb B on mitochondrial apoptosis pathway-related proteins in lung tissues**
The results obtained concerning the effects of Reb B on mitochondrial apoptosis were similar compared to the results of the in vitro cells. WB analysis showed that the Caspase3/b-actin and Bax/Bcl2 level in the LIRI + Reb B significantly reduced compared with the LIRI group (0.54 ± 0.04 vs 0.82 ± 0.03, \( P < 0.001 \) and 0.94 ± 0.07 vs 2.26 ± 0.07, \( P < 0.001 \), respectively, Fig. 5D).

The effect of Reb B on cytokine levels

IL-10 levels were significantly higher in the LIRI+Reb B group compared with the LIRI group (9.39 ± 0.36 vs 7.51 ± 0.58, \( P < 0.05 \)) (Fig. 6C). The levels of IL-1b, IL-8 were significantly lower in the LIRI+Reb B group compared with the LIRI group (8.93 ± 0.26 vs 14.14 ± 0.49, \( P < 0.001 \) and 30.95 ± 0.75 vs 40.40 ± 2.17; \( P < 0.01 \)) (Fig. 6D, E). The levels of TNF-a and IL-6 were not significantly lower in LIRI + Reb B group comparison with LIRI group (55.21 ± 3.42 vs 55.57 ± 3.77, \( P = 0.94 \) and 21.20 ± 0.80 vs 23.65 ± 1.26, \( P = 0.14 \), respectively, Fig. 6F and G)

Discussion

The results presented in the current study suggest that Reb B may exert a significant in vitro and in vivo lung protective effect. To the best of our knowledge, the current study was the first that shows that Reb B inhibits apoptosis in lung tissues and improves lung injury in a simple and validated in vivo LIRI model. Furthermore, Reb B can decrease the number of apoptotic cells and proinflammatory cytokines in lung tissues.

In this context, compounds from natural sources constitute promising candidates in pharmacotherapy to prevent and treat several diseases [9]. Natural compounds have advantages compared with conventional drugs, namely higher efficacy and lower toxicity and for that reason, several natural compounds have been used in clinical practice [10]. High-throughput screening technology facilitates the use of these compound libraries to identify lead compounds that can be further developed into useful therapeutic agents [11]. Human cell lines have been extensively used in high-throughput screening of drugs before animal experiments [12]. Therefore, the human alveolar epithelial cell line A549 that is commonly used to in vitro simulate lung-related diseases was used in this study. Thus, a Natural Product Library that consisted of 2,661 compounds was used, with being Reb B the most promising compound, exhibiting potent anti-apoptotic ability against OGD/R model of A549 cells. Reb B is an important component of SGs, which are an abundant component of Stevia rebaudiana leaf [5]. SGs have become well-known for their intense sweetness (250–300 times sweeter than sucrose) and for that reason are used as a non-caloric sweetener in several countries [13, 14]. Moreover, SGs can inhibit TNF-\( \alpha \) and induce IL-8 release in intestinal cells by inhibiting nuclear factor-kappa B (NF-\( \kappa \)B) activation and suppressing inflammatory cytokines production in lipopolysaccharide (LPS)-stimulated THP-1 cells by interfering with the IKK\( \beta \) and NF-\( \kappa \)B signaling pathway [13, 15, 16]. In this study, the results obtained support that Reb B exerts an anti-apoptotic effect by inhibiting the mitochondrial apoptotic pathway and therefore this pathway was studied in the subsequent in vivo study.
Currently, there is no effective treatment of LIRI. Generally, cell apoptosis involves two major signaling pathways, including the mitochondrial apoptotic pathway and the death receptor apoptotic pathway [17]. Bcl-2 family members regulate the mitochondrial pathway of apoptosis [17]. In LIRI, the levels of Bcl-2 decreased, while the levels of cleaved caspase-3 increased in the lung tissue to activate lung cell apoptosis. The results of this study suggest that administration of Reb B improves physiological function by inhibiting mitochondrial apoptosis pathways in damaged lungs. Neutrophil infiltration and its resulting by-products, such as peroxidases and proteinases can severely damage the lung tissue [18]. The LIRI injury induces the infiltration of neutrophils into the alveoli, which promotes the production of high levels of inflammatory cytokines. The administration of Reb B before LIRI significantly reduces neutrophil infiltration. Regarding the levels of cytokines, the decreased level of IL-1β, IL-8 and increased IL-10 levels in the lung occurred in this study. The level of IL-8 produced correlates with the severity of lung injury [19]. IL-8, a proinflammatory cytokine promotes chemotaxis and degranulation in neutrophils [20]. Furthermore, blocking IL-8 significantly attenuates lung injury caused by ischemia-reperfusion injury [21]. IL-10 is an anti-inflammatory cytokine that controls the inflammatory response [22]. Gene therapy using this cytokine has already been shown to be effective for the treatment of injured donor lungs [23]. Interestingly, IL-6 and TNF-α levels also decreased but without statistical significance.

This study presents several limitations. Thus, although the single dose of Reb B (5 mg/kg) was administered before ischemia, significant improvements were seen in the LIRI. Therefore, more detailed data should be obtained in future studies by performing the experiments with Reb B administration at different doses and frequency of administration. On the other hand, Reb B affects the entire organism and not only the lungs and consequently, the effect of Reb B on the other organs should be further investigated. Male Sprague Dawley rats with a similar age were used in this study, but the potential influence of gender, age, hormone level, and other factors on the results was not studied.

Conclusion

In conclusion, Reb B administration before ischemia attenuated LIRI via inhibition of apoptosis, proinflammatory cytokines production, and neutrophil infiltration in a rat model.

Declarations

Ethics Approval

All procedures carried out in this study were approved by the institutional animal experimental ethics committee of Lanzhou University Second Hospital (Lanzhou, Gansu, China) (No. P2021-019). All experimental procedures involving animals were performed in full agreement with the animal care guidelines of the National Institutes of Health and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Consent for Publication:
Not applicable.

Availability of data and materials:

The data and materials used in the current study are available from the corresponding author on reasonable request.

Competing Interests:

The authors declare no competing interests.

Funding:

This work was supported by the Cuiying Scientific and Technological Innovation Program of Lanzhou University Second Hospital [CY2019-QN01, CY2019-QN12]; the Natural Science Foundation of Gansu Province, China [20JR10RA760, 20JR10RA745, 20JR10RA733, 21JR1RA164]; Innovation Fund for Higher Education of Gansu Province [2020B-037] and the Lanzhou University Second Hospital Talent introduction program in the hospital [YJRCKYQDJ-2021-02].

Author contributions:

BRG, YNL and JH conceived and designed this study. JH, JBY and RZZ and carried out experiments. SXW XDC and JL collected and analyzed data. HJ and SXW performed statistical analysis. HJ wrote the manuscript, which was critically reviewed and revised by BRG and YNL. All authors read and approved the final manuscript.

Acknowledgements:

We thank Bullet Edits Limited for the linguistic editing and proofreading of the manuscript (http://www.bulletedits.cn/). Acknowledgements Figures were created with BioRender (ww.biorender.com).

References


**Figures**
Reb B was identified through high-throughput screening on OGD/R model of A549 cells. The diagram shows the screening approach (A). The chemical structure of Reb B is shown (B). Illustrations were created with BioRender (BioRender.com).
Figure 2

Cell viability of various treatment concentrations of Reb B on OGD/R of A549 cells. In the OGD/R model of A549 cells, the cell viability was the highest when Reb B exposure concentration was 10 mM but gradually decreased with the increase of Reb B concentration (A). Analysis of apoptotic cells staining with Hoechst 33258 in the Vehicle group, OGD/R group, and OGD/R+Reb B group (B) (original magnification 400’). Apoptotic cells were significantly reduced in the OGD/R + Reb B group compared...
with the OGD/R group (C). Quantitative analysis of the mRNA expression of apoptosis-related genes (D). Representative western blot showing changes in the expression of apoptosis-related proteins (E). Quantitative analysis of western blot of apoptosis-related proteins (F). Arrows indicate representative apoptotic-positive cells. Data are presented as mean ± standard error of the mean. ***$P<0.001$, compared with the Vehicle group; ##$P<0.01$, ###$P<0.001$ compared with the OGD/R group.

**Figure 3**

Diagram of the experimental protocol. Three groups of experiments were carried out, and the rats were randomly assigned to the Sham group (n=5), LIRI group (n=6) and LIRI + Reb (n=6).
Figure 4

Representative histologic section of the lungs of the Sham group, LIRI, and LIRI + Reb B group (A). The perivascular edema evaluated using vascular cuff was also ameliorated in the LIRI + Reb B group (B). Arrows indicate representative perivascular edema. Wet-to-dry weight ratio (C). Data are presented as mean ± standard error of the mean. ***$P < 0.001$, compared with the Sham group; ###$P < 0.001$ compared with the LIRI group.
Figure 5

Representative images of TUNEL staining in the Sham group, LIRI group, and LIRI + Reb B group (A). The number of TUNEL–positive cells was significantly lower in the LIRI + Reb B group (C). Representative western blot showing changes in apoptosis-related protein expression in lung tissue (B). Quantitative analysis of western blot of apoptosis-related proteins (D). Arrows indicate representative apoptotic-
positive cells. Data are presented as mean ± standard error of the mean of the mean. ***$P<0.001$, compared with the Sham group; ####$P<0.001$ compared with the LIRI group.

Figure 6

Representative IHC images for Myeloperoxidase in the Sham group, LIRI group and, LIRI+Reb B group (A). The number of myeloperoxidase–positive cells was significantly lower in the LIRI + Reb B group (B). Level
of TNF-a, IL-1b, IL-6, IL-8, and IL-10 in lung tissue lysates of the 3 groups: Sham group, LIRI group, and LIRI + Reb B group (C-G). Arrows indicate representative myeloperoxidase–positive cells. Data are presented as mean ± standard error of the mean of the mean. **P < 0.01, ***P < 0.001, compared with the Sham group; ns represented no significant difference, #P < 0.05, ##P < 0.01 and ###P < 0.001, compared with the LIRI group.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Fig2cleacaspase3.tif
- Fig2Caspase3.tif
- Fig2actin.tif
- Fig5Bax.tif
- Fig2Bax.tif
- Fig5Bcl2.tif
- Fig5Caspase3.tif
- Fig5actin.tif
- FigBcl2.tif