Post-treatment curcumin reduced ischemia–reperfusion-induced pulmonary injury via the Notch2/Hes-1 pathway

Haibo Zou and Xiaofeng Sun

Abstract
Objective: To investigate the influence of curcumin on the Notch2/Hes-1 pathway after pulmonary injury induction via limb ischemia–reperfusion (I/R).
Methods: Adult male Sprague–Dawley rats were randomly divided into four groups (n = 30 each): sham, I/R, curcumin post-treatment (I/R+Cur), and inhibitor (I/R+DAPT). Hind-limb ischemia was induced for 4 hours, followed by reperfusion for 4 hours. After ischemia, curcumin (200 mg/kg) or DAPT (0.5 mm) was injected intraperitoneally in the I/R+Cur or I/R+DAPT group, respectively. PaO2 was examined after 4 hours of reperfusion. Pathological changes in the lung and the apoptotic index (AI) were examined. Lung malondialdehyde (MDA), tumor necrosis factor (TNF)-α, and interleukin (IL)-1β levels, the wet/dry (W/D) ratio, semi-quantitative score of lung injury (SSLI), and Notch2 protein and Hes1-mRNA expression were also examined.
Results: In the I/R group, inflammatory changes were observed, AI increased, and MDA, SSLI, W/D, TNF-α, IL-1β, Notch2, and Hes1-mRNA expression increased, while PaO2 decreased compared with the Sham group. Pathological changes in the I/R+Cur group were reversed. All indexes in the I/R+DAPT and I/R+Cur group were similar.
Conclusion: Curcumin post-treatment reduced I/R-induced lung injury in rats. Its mechanism may be related to the inhibition of Notch2/Hes-1 signaling pathway and the release of inflammatory factors.

Keywords
Curcumin, post-treatment, Notch2/Hes-1, hind leg, ischemia–reperfusion, pulmonary

Date received: 6 July 2019; accepted: 12 November 2019
Introduction

In addition to limb injury, ischemia/reperfusion (I/R) can also cause functional and organic pathological damage to distant organs, especially the blood-rich lung, and it can cause conditions such as acute respiratory distress syndrome (ARDS). Studies have shown that limb I/R is a complex process that involves multiple signaling pathways and inflammatory factors. Curcumin is a phenolic pigment that is extracted from the rhizome of Curcuma longa, and it is a main active ingredient with strong anti-inflammatory and antioxidant effects. The Notch signaling pathway exists widely in vertebrates and non-vertebrates, and it is highly conserved in evolution. Both humans and rats have four Notch receptors. Previous studies have shown that curcumin pre-treatment can alleviate lung injury that is induced by limb I/R through anti-inflammatory and anti-oxidative effects. Other recent studies from our group indicated that curcumin post-conditioning could be effective methods against renal injuries, which were induced by limb I/R in rats via the Notch2/Hes1 pathway. However, it is unclear whether curcumin post-treatment has a protective effect on lung injury that is induced by limb I/R. We suggest that the post-conditioning with curcumin may also play a protective role in lung injury that is induced by limb I/R in rats through the Notch2/Hes-1 signaling pathway. However, it is unclear whether curcumin post-treatment has a protective effect on lung injury that is induced by limb I/R in rats. In future clinical trials, this study aims to further explore the effect of curcumin post-treatment on lung injury induced by limb I/R in rats.

Materials and methods

Major materials

The following reagents and equipment were purchased from the respective suppliers: Curcumin (batch number: 86M1611V, Sigma-Aldrich, St Louis, MO, USA); ES-1000 SPM Ultrasound Blood Flow Meter (Hayashi Denki, Osaka, Japan); Gem Premier 3000 Blood Gas Analyser (Instrumentation Laboratory Company, Bedford, MA, USA); DYY-6B Electrophoresis Meter (Beijing Liuyi Instrument Factory, Beijing, China); Eppendorf AG polymerase chain reaction (PCR) amplifier (Hamburg, Germany); BX-41 Microscope (Olympus Corporation, Tokyo, Japan); alkaline phosphatase-labeled antibody II (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Scion Image Analysis System (Apple, Cupertino, CA, USA); Trizol method (Invitrogen, Carlsbad, CA, USA); Hes1-RNA primer (Bao Bioengineering Co. Ltd., Dalian, China); rabbit anti-rat Notch2 polyclonal antibody and rabbit anti-rat beta-act polyclonal antibody (Abcam Co., Millbrae, CA, USA); biotinized antibody working solution and end stop solution (Beijing Qinbang Biotechnology Co. Ltd., Beijing, China); horseradish peroxidase (HRP) solution (Psaitong Co., Beijing, China); and Curve Expert software (Beijing, China).

Animals and experimental groups

In these experiments, 120 adult male SD rats were used (280–320 g, 6 to 8 months old) and they were randomly divided into the following four groups (n = 30): sham, I/R, curcumin post-conditioning (I/R+Cur), and inhibitor (IR+DAPT) groups. The protocol was approved by the Ethics Committee of Central Hospital that is affiliated with Shenyang Medical College (approval No: SYXK(liao)201900007).

Ischemia–reperfusion model

The model of lung injury induced by limb I/R in rats was established based on a
previously published study. The rats were fasted for 12 hours with free access to water before the experiment was performed. Under 3% sodium pentobarbital (40 mg/kg intraperitoneally) anesthesia, access to the right external jugular vein was established. The skin was cut in the femoral triangle of both hind limbs, and the femoral artery and vein were separated. The femoral artery was clamped near the inguinal ligament using a non-invasive microartery clamp. After ischemia of both hind limbs for 4 hours, the non-invasive microartery clamp was loosened and reperfusion occurred for 4 hours. Blood flow was monitored using an ES-1000 SPM ultrasonic blood flow meter (Hayashi Denki, Osaka, Japan). No blood flow was considered to indicate successful ischemia and the blood flow was monitored as a successful criterion for reperfusion. During the experiment, saline was infused intravenously (1.5 mL/kg/hour).

The femoral artery and vein were separated without clamping in the Sham group. The limb I/R model was established in the I/R, I/R+Cur, and I/R+DAPT groups. In the I/R+Cur group, curcumin was immediately injected (200 mg/kg, dissolved in 2 mL saline) after ischemia for 4 hours. In the I/R+DAPT group, DAPT (a γ-secretase inhibitor that has an inhibitory effect on the Notch2/Hes-1 signaling pathway) was immediately injected (0.5 μm, dissolved in 2 mL saline) after ischemia for 4 hours. The Sham and I/R groups were administered the same amount of saline.

At 4 hours after reperfusion, 3 mL of the arterial blood was collected from the carotid artery. An arterial blood gas analysis was immediately performed using a Gem Premier 3000 blood gas analyzer (Instrumentation Laboratory Co.). PaO₂ was recorded. The rats were then sacrificed by exsanguination. The upper lobe of right lung was taken for 1 cm³ to examine the wet/dry (W/D) ratio. The residual blood was washed out with saline at 4°C. The wet weight (W) was measured after treatment and absorption with the filter paper, and the lung tissue was dried at 80°C for 48 hours. The W/D ratio of lung was then calculated. The middle and lower lobes of the right lung (1 cm³) were used to examine Notch2 protein and Hes-1 mRNA expression in the 10% lung tissue homogenate. The upper and middle pole of the left lung were taken (1 cm³) to examine tumor necrosis factor (TNF-α), interleukin (IL)-1β, and malondialdehyde (MDA) in the 10% tissue homogenate. The lower pole of the left lung was fixed in 10% neutral formalin, and it was embedded with paraffin and made into sections for hematoxylin and eosin (HE) and Hoechst 33258 staining. Pathological changes in the lung tissue were observed using a BX-41 microscope (Olympus Corporation). The Smith scoring method was used to analyze pulmonary edema, alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, atelectasis, and hyaline membrane formation using a 0 to 4-point scale, as follows: 0, no injury; 1, lesion range was less than 25%; 2, lesion range was 25% to 50%; 3, lesion range was 50% to 75%; and 4, lesion range was greater than 75%. Ten high-magnification fields of lung tissue slices were observed randomly in each rat. The semi-quantitative score of lung injury (SSLI) was the sum of the above scores.

**Notch2 protein expression**

The western blot method was used. Total protein was extracted from 10% lung homogenate by centrifugation and quantified. After SDS polyacrylamide gel electrophoresis separation, transformation, and closure, rabbit-anti-rat Notch2 polyclonal antibody and rabbit-anti-rat β-actin polyclonal antibody were added, and then incubated at 4°C overnight. Alkaline phosphatase-labeled antibody was added
and incubated at room temperature for 2 hours. Enzyme was added for color, and then scanned. The Scion Image analysis system (Apple) was used to analyze Notch2 protein expression. The gray value ratio of the target product and β-actin was used to reflect Notch2 protein expression.

**Hes-1 mRNA expression**

Real time (RT)-PCR was used. Total RNA was extracted from 10% lung tissue homogenate using the Trizol method, and reverse transcription was used to synthesize the cDNA. DNA amplification was then performed. The sequence of primers was as follows: Hes-1 mRNA upstream primer 5'-AGAGAGGCGGCTCCGACGG-3' and downstream primer 5'-TTGGATGGTGCACTGGATTCCG-3'; and β-actin upstream primer 5'-GCGAGGAGAGTACG-3' and downstream primer 5'-GGACACAGAGGAGC-3'. The reaction conditions were as follows: 35 cycles of pre-denaturation at 94°C for 2 minutes, 95°C for 45 seconds, 57°C for 45 seconds, and 72°C for 60 seconds; followed by extension at 72°C for 5 minutes. The β-actin reaction conditions were as follows: 30 cycles of pre-denaturation at 94°C for 2 minutes, 94°C for 40 seconds, 58°C for 40 seconds, and 72°C for 60 seconds; followed by extension for 5 minutes. The products were analyzed using 2% agarose gel electrophoresis, ethidium bromide staining, and a gel imager. Hes-1 mRNA expression was reflected by the gray value ratio of the target product and β-actin using the Scion Image analysis system.

**TNF-α and IL-1β levels**

The 10% lung homogenate was analyzed using ELISA. The TNF-α standard was diluted using a multiple gradient, and then added into the detection wells (100μL/well). The supernatant was collected and then added into the well (100μL/well). The plates were sealed and then incubated for 2 hours at 37°C in an incubator. The wells were then washed and biotinylated antibody working solution (100μL/well) was added. The plates were sealed and incubated at room temperature for 1 hour followed by washing. HRP solution was added (100μL/well) and incubated at room temperature for 20 minutes. Each well was then washed and color reagent was added (100μL/well), followed by incubation for 20 minutes in the dark. Termination solution (50μL/well) was then added followed by gentle shaking, and the labeled enzyme was then added to measure the optical density (OD) value at 450 nm. The OD value of a blank sample was calculated and the average value was taken as the measurement value. The standard curve was made using Curve Expert software, and the corresponding OD value was input to calculate the TNF-α concentration. Similarly, IL-1β was detected.

**Detection of pulmonary epithelial cell apoptosis**

The specimen was analyzed by routine sectioning and embedding and washed with phosphate buffered saline (PBS). The specimen was immersed in Hoechst 33258 staining solution (0.5 mL) for 5 minutes and then washed with PBS solution. The sample was then placed onto a slide, and anti-quenching sealing solution was dropped onto it. The slide was covered and observed under 400× magnification using a fluorescence microscope. Microscopically, the nuclei of normal cells were round and dark blue. However, the chromatin of apoptotic cells was condensed, and the nuclei were densely stained, while the cells turned a bright white color. Each slice was randomly divided into five visual fields, and 100 cells were counted in each visual field. The apoptotic index (AI) was expressed as the proportion of apoptotic cells per 100 cells in the lung.
Statistical analysis

SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data that were not normally distributed were presented as the mean ± standard deviation (x ± s). A one-way analysis of variance (ANOVA) and the least significant differences (LSD) post-hoc test were used to compare between the groups. Additionally, p < 0.05 was considered to be statistically significant.

Results

HE staining

Compared with the Sham group, the number of inflammatory cells in the visual field increased in the samples that were stained with HE, capillaries were congested and dilated, and the alveolar septum and lung interstitium were thickened in the I/R group (p<0.05). Inflammatory infiltration into the pulmonary interstitium and some localized atelectasis were also observed in the I/R group. Compared with the I/R group, HE staining in the I/R + Cur group showed no localized atelectasis, and only a small amount of inflammatory infiltration in pulmonary alveoli and interstitium. The changes in the I/R + Cur and I/R + DAPT groups were similar compared with the I/R group, as shown in Figure 1.

W/D, SSLI, and PaO₂

Compared with the Sham group, W/D and SSLI increased (p<0.05) and PaO₂ decreased (p<0.05) in the I/R group. Compared with the I/R group, W/D and SSLI decreased (p<0.05) and PaO₂ increased significantly (p<0.05) in the I/R + Cur group. There was no statistically significant difference between the I/R + Cur and I/R + DAPT groups, as shown in Table 1.

TNF-α, IL-1β, AI, and MDA

Compared with the Sham group, the TNF-α, IL-1β, and MDA levels and the AI increased in the I/R group (p<0.05). Compared with the I/R group, all of these factors decreased in the I/R + Cur group (p<0.05). There was no statistically significant difference between the I/R + Cur and I/R + DAPT groups, as shown in Table 2 and Figure 2.

Notch2 protein and Hes-1 mRNA expression

Compared with the Sham group, Notch2 protein expression and Hes-1 mRNA expression increased in the I/R group (p<0.05). Compared with the I/R group, Notch2 protein expression and Hes-1 mRNA expression decreased in the I/R + Cur group (p<0.05). There was no statistically significant difference between these expression levels in the I/R + Cur and the I/R + DAPT groups, as shown in Table 3 and Figures 3 and 4.

Discussion

As the main manifestation of the pulmonary gas-exchange function, partial pressure of oxygen is a sensitive index for evaluating the degree of lung injury and its protective effect. Compared with the Sham group, the I/R group showed inflammatory cell infiltration, alveolar septum thickening, localized atelectasis, and other obvious inflammatory changes in the sections. Additionally, the SSLI and the W/D ratio were significantly increased, and arterial blood gas analysis indicated that PaO₂ was decreased in the I/R group compared with the Sham group. The above results showed that the model of lung injury induced by limb I/R was successfully established in the rats.
Curcumin is a chemical constituent that is extracted from the rhizomes of some plants, such as the Zingiberaceae family and the Araceae family. Curcumin is a phenolic compound that is the basic pigment of the *Curcuma longa* plant, which has anti-inflammatory, antioxidant, and anti-carcinogenic effects. Studies have shown that curcumin has a wide range of pharmacological activities such as reducing oxidative stress, inhibiting the release of inflammatory cytokines, and anti-apoptosis. Among them, the anti-inflammatory and anti-oxidative effects of curcumin have been widely recognized by researchers worldwide.

Notch genes encode a highly conserved class of cell surface receptors that regulate...
the development of many biological cells. Notch signaling affects many processes of normal cell morphogenesis, including cell apoptosis, cell proliferation, and cell boundary formation. The Notch signaling pathway exists widely in vertebrates and non-vertebrates and it is highly conserved in evolution. Notch, a transmembrane protein that was first found in flies, is ideally suited to precisely regulate cell-to-cell communication during development of complex tissues such as the lung.¹⁰

Previous studies have confirmed that curcumin preconditioning has a protective

Table 2. Detection of TNF-α, IL-1β, AI, and MDA in lung tissues of each group (n = 20, x ± s).

| Detection Index | Sham Group | I/R Group | I/R+Cur Group | I/R+DAPT Group |
|-----------------|------------|-----------|---------------|---------------|
| TNF-α (mg/mL)  | 1.15 ± 0.03 | 2.87 ± 0.13<sup>d</sup> | 1.59 ± 0.09<sup>e</sup> | 1.47 ± 0.11<sup>f</sup> |
| IL-1β (ug/L)   | 0.18 ± 0.03 | 0.45 ± 0.05<sup>d</sup> | 0.21 ± 0.02<sup>e</sup> | 0.18 ± 0.07<sup>f</sup> |
| AI (%)         | 35.68 ± 3.07 | 54.12 ± 2.78<sup>d</sup> | 39.45 ± 4.38<sup>e</sup> | 37.53 ± 2.57<sup>f</sup> |
| MDA (U/mg prot)| 5.93 ± 0.37 | 16.68 ± 0.78<sup>d</sup> | 9.16 ± 0.54<sup>e</sup> | 8.16 ± 0.23<sup>f</sup> |

Note: Compared with the Sham group, <sup>d</sup>p < 0.05; compared with the I/R group, <sup>e</sup>p < 0.05; compared with the I/R+Cur group, <sup>f</sup>p > 0.05.
I/R, ischemia–reperfusion; Cur, curcumin; DAPT, γ-secretase inhibitor that has an inhibitory effect on the Notch2/Hes-1 signaling pathway; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; AI apoptotic index; MDA, malondialdehyde.

Figure 2. Comparison of apoptosis in pulmonary epithelial cells in each group (Hoechst 33258; magnification, ×40).
effect against lung injury that is induced by limb I/R in rats, but the effect of administering curcumin post-treatment on this lung injury in rats is still uncertain. Our previous studies indicated curcumin post-conditioning could play a protective role in the lung injuries induced by limb I/R via inhibiting the TRL4/NF-κB p65 pathway. Our previous research results also showed similar protective effects in the rat kidney, as assessed using the matrix metalloproteinase (MMP)-9/ tissue inhibitors of metalloproteinase (TIMP)-1 ratio during the process of limb I/R, but the protective

Table 3. Notch2 and Hes-1 mRNA expression in lung tissues of each group (n = 20, x ± s).

| Detection Index | Sham Group | I/R Group | I/R + Cur Group | I/R + DAPT Group |
|-----------------|------------|-----------|----------------|-----------------|
| Notch2 protein  | 0.42 ± 0.03| 1.69 ± 0.27<sup>a</sup> | 0.71 ± 0.33<sup>b</sup> | 0.58 ± 0.12<sup>i</sup> |
| Hes-1 mRNA      | 0.19 ± 0.02| 1.21 ± 0.09<sup>b</sup> | 0.44 ± 0.03<sup>b</sup> | 0.39 ± 0.07<sup>i</sup> |

Note: Compared with the Sham group, <sup>a</sup>p<0.05; compared with the I/R group, <sup>b</sup>p<0.05; compared with the I/R + Cur group, <sup>i</sup>p>0.05.

I/R, ischemia–reperfusion; Cur, curcumin; DAPT, γ-secretase inhibitor that has an inhibitory effect on the Notch2/Hes-1 signaling pathway.

Figure 3. Notch2 protein expression in the lung tissue of each group.

Figure 4. Hes-1 mRNA expression in the lung tissue of each group.
effects in the rat lung via the Notch2/Hes-1 signal pathway were not reported. In our experiment, inflammatory cell infiltration, alveolar septum thickening, alveolar interstitial congestion and edema, and occasional localized atelectasis were observed in the lung HE pathology section in the I/R group. Further studies showed that Notch2 protein and Hes-1 mRNA expression increased in the I/R group. Inflammatory cytokine release was detected by ELISA. Studies have shown that most of the organs may be protected from the damaging effects of the reactive oxygen species by enzymatic and nonenzymatic antioxidant defense mechanisms. MDA is a good indicator of free radical activity and the increasing presence of lipid peroxidation. The lung is susceptible to systemic inflammatory responses. A large number of oxygen free radicals can activate inflammatory factors, and TNF-α and IL-1β are the main cytokines that initiate the inflammatory response.

The above experimental results showed that limb I/R activated the Notch2/Hes-1 signaling pathway. After Notch2 activation, Hes-1, which is located downstream, was up-regulated. Then, Hes-1 stimulated the release of inflammatory cytokines, which eventually led to lung injury. However, curcumin post-treatment significantly reduced alveolar injury, such as infiltration of inflammatory cells, congestion of the alveolar septum, and alveolar exudation. Localized atelectasis disappeared. Western blot and RT-PCR were used to detect Notch2 protein expression and Hes-1 mRNA expression, respectively, in lung tissue. Compared with the I/R group, experimental results also showed that TNF-α, IL-1β, and MDA levels and the AI, W/D, and SSLI results were much lower compared with the I/R+Cur group. These results suggest that curcumin post-treatment might play a protective role in lung injury induced in rats by down-regulating Notch2/Hes-1 signaling pathway. Based on the above experimental results, limb I/R injury activates the Notch2/Hes-1 signaling pathway and leads to distant lung injury, but curcumin post-conditioning can significantly down-regulate this pathway. We also used DAPT, a specific inhibitor of Notch2/Hes-1 signaling, which is the gold standard for inhibition, to further evaluate the extent of these protective effects.

Conclusion
Curcumin post-treatment can reduce the lung injury that is caused by hind-leg I/R injury in rats, and its mechanism may be related to the inhibition of the Notch2/Hes-1 signaling pathway, the release of the inflammatory factors TNF-α, IL-1β, and MDA, and the AI. The potency of curcumin post-treatment is almost equivalent to that of DAPT (0.5 μm).

Acknowledgement
We thank the teachers at the Central Hospital Affiliated to Shenyang Medical College and the Animal Laboratory Center of China Medical University for their strong support during the experiment and for the encouragement given by the leaders at the hospital and in the department. In addition, we would like to thank all the experimenters and data collectors who participated in the conduct of the experiment, as well as the statisticians for their help.

Consent
All of the authors declare that once this article had been accepted by the editorial office of JIMR, and the journal has the right to use and publish the article.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.
Ethics
The Ethics Committee of the Affiliated Central Hospital of Shenyang Medical College approved the experiments (Ethics Committee License Number: SYXK(liao)2013-0007).

Funding
Funding was received from the following: Research fund for Science-Technology of Shenyang Medical College NO: 20191013; Project fund for Science and Technology of Liaoning Province in China (NO: 20180550621); General Projects of Liaoning Provincial Department of Education in China (NO: L2015540); and Youth Foundation Project of Shenyang Medical College (NO: 20162029).

ORCID iD
Haibo Zou https://orcid.org/0000-0002-8442-8113

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