Resveratrol attenuates spinal cord injury-induced inflammatory damage in rat lungs

Jia Liu1,2, Long Yi2, Zimin Xiang2, Jianfeng Zhong2, Hao Zhang2, Tiansheng Sun2

1Department of Medical School of Chinese PLA, Beijing 100583, China; 2Department of Institute of Orthopaedics, Chinese PLA (People’s Liberation Army) Beijing Army General Hospital, Dongcheng District, Nanmencang Number 5, Beijing 100700, China

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Abstract: Spinal cord injury (SCI)-induced systemic inflammatory response affects multiple organs outside the spinal cord. Treatment options for such complications are lacking. We studied the potential protective effects of resveratrol on SCI-induced inflammatory damage in rat lungs. Sprague-Dawley rats were subjected to weight-drop impact at the T10 vertebral level with administration of resveratrol (100 mg/kg) or vehicle (via the intraperitoneal route) immediately after trauma. Lung injury was studied by measuring: vascular permeability-related pulmonary edema; histopathologic scores, neutrophil infiltration and concentrations of inflammatory cytokines in bronchoalveolar fluid; expression of inflammatory enzymes and sirtuin (SIRT) 1 as well as nuclear factor-kappa B (NF-κB) activity in pulmonary tissues. Resveratrol treatment significantly alleviated SCI-induced pulmonary edema as indicated by the ratio of the wet weight to dry weight of lung tissue and pulmonary permeability index. Resveratrol significantly reduced neutrophil infiltration and production of inflammatory mediators. Resveratrol treatment was accompanied by up-regulation of expression of SIRT1 and suppression of NF-κB activity in pulmonary tissues. These data suggest that resveratrol may protect the lungs from SCI-induced inflammatory damage, and could be used as a therapeutic option against pulmonary problems after SCI.

Keywords: Resveratrol, spinal cord injury, rat, acute lung injury, inflammatory response

Introduction

Traumatic spinal cord injury (SCI) is a disastrous event associated with high morbidity and mortality [1]. Epidemiologic investigations have shown that pulmonary complications (especially pulmonary infection and respiratory failure) are the leading cause of death in patients with acute SCI [2, 3]. For many years, satisfactory treatment was not available because of a lack of clear understanding of the pathogenesis of pulmonary complications after SCI (especially injury to the lower thoracic spinal cord (LTSC)) [4]. Therefore, many efforts have been made to explore the mechanisms underlying pulmonary events after SCI, and to seek optimal treatment.

In recent years, the systemic inflammatory response has gained much attention as an important contributory factor in the development of SCI-induced pulmonary dysfunction [5, 6]. Gris et al. stated that a considerable number of circulating leukocytes infiltrate the lungs after SCI and result in acute lung injury (ALI), and that this phenomenon is allied with over-expression of pro-inflammatory mediators [6]. Moreover, subsequent studies demonstrated that inflammatory damage to the lungs after SCI might be alleviated by blocking the onset of the systemic inflammatory response [7, 8]. These results suggest that anti-inflammatory agents could be used to alleviate ALI and the pulmonary complications after traumatic SCI.

Resveratrol (3,4’; 5-trihydroxystilbene) is a natural polyphenol found in red grapes, peanuts and mulberries [9] which has anti-oxidation [10], anti-tumor [11] and anti-inflammation [12] activities. Studies have shown that the anti-inflammatory effects of resveratrol are mediated primarily by sirtuin (SIRT) 1 [13, 14]. SIRT1 activation leads to inhibition of expression of nuclear factor-kappa B (NF-κB) and subsequent
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Reduction in production of pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF) [15, 16]. Recently, it was reported that resveratrol can protect the lungs from damage induced by staphylococcal enterotoxin B or lipopolysaccharide (LPS) [17, 18]. However, reports about the protective effect of resveratrol on SCI-induced ALI are lacking.

We created a rat model of traumatic SCI. Then, we investigated the protective effects of resveratrol on SCI-induced ALI in rat lungs and the possible underlying mechanisms of action. Our data demonstrated that resveratrol markedly inhibited lung inflammation after SCI by reducing leukocyte infiltration and reducing excessive secretion of pro-inflammatory factors through mediation of inflammatory related pathways, and subsequent alleviation of histologic changes in the lungs.

Materials and methods

Animals and experimental groups

All experiments were carried out in accordance with the guidelines established by the Animal Ethics Committee of Beijing Military General Hospital (Beijing, China). Adult female Sprague-Dawley rats (220-250 g; Beijing Haidian Thriving Experimental Animal Centre, Beijing, China) were housed in a standard Animal Room and fed using commercial rodent chow. Eighty rats were allocated randomly into four groups: uninjured; SCI; SCI + vehicle (veh); (3) SCI + resveratrol (RES).

SCI

Animals were anesthetized using 10% chloral hydrate (3.0 mg/kg body weight, i.p.). A contusive model of SCI was established as described previously [19]. Briefly, after a thoracic-level (T8-T11) midline skin incision and dissection of paravertebral muscle, laminectomy of T10 was undertaken to expose the dorsal surface of the spinal cord without disrupting the dura. Then, rats were subjected to a 25 g/cm impact (10 g x 2.5 cm). After trauma, the incision was closed, and rats were allowed to recover from anesthesia in a warm box. Twelve hours after SCI induction, animals were killed and specimens collected.

Pharmacologic treatment

Immediately after trauma, rats in the SCI + RES group were administered a single dose of resveratrol (100 mg/kg body weight, i.p.; Sigma-Aldrich, Saint Louis, MO, USA) in a volume of 1 mL. Rats in the SCI + Veh group were given 1 mL physiologic (0.9%) saline via the intraperitoneal route instead of resveratrol. A further 20 rats who did not undergo weight-drop trauma and administration of medication were used as the uninjured group.

Ratio of wet weight to dry weight (W/D) of lung tissue and measurement of the pulmonary permeability index (PPI)

The W/D ratio of lung tissues and the PPI were assessed according to the method described by Gao et al. [20]. Briefly, lungs were removed and the wet weight recorded. Obtained lung tissue was placed in an oven at 60°C for 72 h and then weighed to obtain the dry weight. The W/D ratio was used to assess the extent of lung edema (n = 4 for each group).

The protein level in the collected bronchoalveolar fluid (BALF) and plasma were determined using a Bicinchoninic Acid (BCA) Protein Assay kit (Pierce, Rockford, IL, USA). The BALF/plasma protein ratio (which is also known as the PPI) was used to evaluate vascular permeability in the airways (n = 4 for each group).

Histologic analyses of lung tissue

For histologic analyses, deep anesthesia was induced in rats. Rats were then perfused with 0.9% saline for 12 h after contusive injury followed by 300 mL of 4% paraformaldehyde (n = 4 for each group). Lungs were perfused separately via the pulmonary artery and removed. After post-fixing, the left lung was cryoprotected in increasing concentrations of sucrose and cut into slices (thickness, 10 μm) with a cryostat.

Sections were stained with hematoxylin and eosin (H & E) and scored by a pathologist blinded to the experimental groups. Histologic scoring was undertaken as described previously [21] based on categories of infiltration of inflammatory cells, edema, congestion, and intra-alveolar hemorrhage: 0: normal; 1: mild; 2: moderate; 3: severe injury. The maximum possible score was 12.
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Measurement of levels of inflammatory cytokines in BALF and sera

Concentrations of TNF-α, interleukin (IL)-6 and IL-10 in sera as well as supernatants of BALF were determined using enzyme-linked immunosorbent assay (ELISA) kits specific for rats (R & D Systems, Minneapolis, MN, USA) according to manufacturer instructions. Finally, the optical density (OD) of the microplate was read at 450 nm.

Assay to measure myeloperoxidase (MPO) activity in lungs

MPO is a marker of neutrophil sequestration [22]. MPO activity in lungs was determined using a commercial kit (Jiancheng, Nanjing, China) according to manufacturer instructions and expressed as units/g tissue (n = 4 for each group).

Western blot

Twelve hours after SCI, rats were perfused via a transcardial approach with 300 mL of ice-cold 0.9% saline, and the lungs were perfused separately via the pulmonary artery again. The left lung was removed and stored at -80°C. Some specimens were homogenized in RIPA buffer and centrifuged at 12,000 rpm for 30 min at 4°C. Protein concentration in the supernatant was determined by the BCA method. Membranes were blocked with 5% skimmed milk and incubated overnight at 4°C with primary antibodies for phosphorylated NF-κB (1:1000

Figure 1. Resveratrol alleviated pulmonary edema after SCI. Mean values of the lung W/D ratio (A) and PPI (B) of rats from uninjured, SCI, SCI + Veh and SCI + RES groups. (C) Western blot analyses of APQ1 level in the rat lungs of different groups 12 h after SCI. **P < 0.001 vs. uninjured group, ***P < 0.001 vs. SCI group.
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Figure 2. Resveratrol alleviated histologic damage in the lungs 12 h after SCI. H & E-stained pulmonary histologic sections of rats from different groups (A). Scale bar = 50 μm. Photograph of lung tissue obtained from a rat in the SCI group showed increased congestion, pulmonary edema, neutrophil infiltration (arrows), and disrupted alveolar architecture. (B) Calculated lung-injury scores of different groups. **P < 0.001 vs. uninjured group, ##P < 0.001 vs. SCI group.

Figure 3. Resveratrol inhibited MPO activity in lungs after SCI. Mean values of MPO level in pulmonary tissues from uninjured rats and rats subjected to SCI, treated with veh or reseratrol 12 h after SCI. **P < 0.001 vs. uninjured group, ##P < 0.001 vs. SCI group.

dilution; Cell Signaling Technology, Danvers, MA, USA), SIRT1 (1:1000; Millipore, Bedford, MA, USA), iNOS (1:1000; Millipore), cyclooxygenase-2 (COX-2; 1:1000; Millipore), aquaporin-1 (APQ-1; 1:1000; Millipore), cleaved caspase-3 (1:1000; Cell Signaling Technology) and β-actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After five washes in TBST, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology) for 1 h at 37°C. Protein bands were detected using enhanced chemiluminescence (Thermo Scientific, Waltham, MA, USA), and assessed for protein levels using Quantity One (Bio-Rad, Hercules, CA, USA) (n = 4 for each group).

Statistical analyses

Data are the mean ± SEM and were analyzed with SPSS v13.0 (IBM, Armonk, NY, USA). Comparison between groups was made with one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. For all analyses, P < 0.05 was considered significant.

Results

Effects of resveratrol on lung edema after SCI

Twelve hours after SCI, the W/D ratio (Figure 1A) and PPI (Figure 1B) of lungs were evaluat-
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Effects of resveratrol on SCI-induced histologic changes in the lungs

To evaluate the protective effect of resveratrol on indirect lung injury after SCI, we also observed histological changes in the lungs using electronic light microscopy 12 h after surgery. H & E-stained lung sections in the SCI group showed significant histologic changes, which presented with increased congestion, pulmonary edema, over-infiltration of neutrophils, and disrupted alveolar architecture ($P < 0.05$, Figure 2A). Compared with the SCI group, morphologic changes were alleviated significantly in the SCI + RES group ($P < 0.05$). Calculated lung-injury scores are shown in Figure 2B.

Figure 4. Resveratrol reduced production of pro-inflammatory cytokines and increased levels of anti-inflammatory cytokine IL-10 in BALF and sera after SCI. Mean values of concentrations of TNF-$\alpha$, IL-6 and IL-10 in BALF (A) and sera (B) from rats without trauma and rats subjected to SCI, treated with veh or resveratrol 12 h after SCI. **$P < 0.001$ vs. uninjured group, *$P < 0.05$ vs. SCI group.

To ascertain if development of SCI-induced ALI was also accompanied by changes in AQP1 expression, we also measured levels of AQP1 protein by western blotting. SCI induced a significant reduction of AQP1 expression in the lungs and resveratrol treatment could reverse this change ($P < 0.05$, Figure 1C).

Effects of resveratrol on SCI-induced histologic changes in the lungs

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Effects of resveratrol on MPO activity

MPO activity was determined to assess neutrophil accumulation within pulmonary tissues. MPO activity of lung tissues increased significantly in the SCI group compared with that of the uninjured group \((P < 0.05, \text{ Figure } 3)\). However, this increase was reduced by resveratrol treatment \((P < 0.05)\).

Effects of resveratrol on levels of inflammatory cytokines and enzymes after SCI

ELISA results showed that trauma to the spinal cord caused significant elevation in levels of TNFα, IL-6 and IL-10 in BALF and sera \((P < 0.05)\). Resveratrol treatment significantly reduced the protein concentrations of pro-inflammatory cytokines including TNFα and IL-6, and further increased the concentration of anti-inflammatory cytokine IL-10 \((P < 0.05, \text{ Figure } 4)\).

We also evaluated expression of critical pro-inflammatory enzymes such as iNOS and COX-2 in lungs 12 h after SCI. SCI caused a significant increase in expression of iNOS and COX-2 proteins in the SCI group \((P < 0.05)\). However, this increase was markedly down-regulated upon intraperitoneal injection of resveratrol in the SCI + RES group \((P < 0.05, \text{ Figure } 5)\).

Effects of resveratrol on SIRT1 expression, and activation of caspase-3 and NF-κB

NF-κB plays a central part in the inflammatory response, activation of which can be mediated by SIRT1 \([15]\). We measured levels of SIRT1 and phosphorylated nuclear factor-kappa B (p-NF-κB) in the lungs 12 h after SCI. Analyses of data from western blotting demonstrated that SCI triggered slightly up-regulated expression of SIRT1 and increased p-NF-κB levels significantly \((P < 0.05)\). However, resveratrol treatment caused a further significant up-regulation of SIRT1 expression and decreased activation of NF-κB in the lungs \((P < 0.05, \text{ Figure } 6)\).

To ascertain if alleviation of histologic changes is accompanied by reduction of apoptosis, we measured levels of cleaved caspase-3 protein in the lungs. Caspase-3 was activated to a lesser extent in the SCI + RES group than in the SCI group \((P < 0.05, \text{ Figure } 7)\).

Discussion

Clear understanding of the pathogenesis of pulmonary complications in the early stage of the SCI (especially of injury to the LTSC) is lacking \([24]\). We investigated the pathologic consequences in pulmonary tissue using a T10 contusive rat model. Our results showed that trauma to the T10 segment evoked severe pulmonary edema and inflammation, a result that is consistent with the report of Nan et al. \([25]\). Moreover, we demonstrated that resveratrol might limit SCI-induced pathologic processes in the lungs via inhibition of inflammation.

Pulmonary edema is a well-known characteristic of ALI and the main factor contributing to pulmonary complications in injury to the LTSC.
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Studies have shown that increased microvascular leakage and decreased APQ1 levels are closely associated with impaired transport of fluids, and contribute significantly to the development of pulmonary edema [28, 29]. We measured the W/D ratio of the lungs to quantify the magnitude of SCI-induced pulmonary edema. We also measured microvascular leakage and APQ1 levels to further explore the mechanisms underlying pulmonary edema. Trauma to the T10 segment led to significantly increased capillary leakage, down-regulated expression of APQ1, and subsequent increased W/D ratio in the lungs. More importantly, we also found that resveratrol might significantly attenuate pulmonary edema, which suggested that resveratrol treatment had a strong protective effect on SCI-induced ALI.

Pro-inflammatory cytokines such as TNFα and IL-6 have pathogenic roles in the progression of ALI [30]. The key pro-inflammatory enzymes iNOS and COX-2 also contribute to the development of lung inflammation through promotion of production of inflammatory mediators [31]. In our SCI-induced model of ALI, we detected increased protein levels of pro-inflammatory and anti-inflammatory cytokines in sera and BALF as well as pro-inflammatory enzymes in lung tissues. Moreover, we found that resveratrol treatment significantly reduced the levels

Figure 6. Resveratrol increased expression of Sirt1 and increased activation of NF-κB in lungs after SCI. A. Western blot analyses of expression of Sirt1, p-NF-κB and β-actin protein. B. Quantitative analyses of western blots showed that resveratrol treatment significantly reduced expression of pNF-κB accompanied by a further increase in Sirt1 level compared with those of the SCI group. **P < 0.001 vs. uninjured group, #P < 0.05 vs. SCI group.

Figure 7. Resveratrol inhibited apoptosis in lungs after SCI. A. Western blot analyses of expression of cleaved caspase-3 and β-actin protein. B. Quantitative analyses of western blots showed that levels of cleaved caspase-3 in resveratrol-treated rats were significantly lower than those in the SCI group. **P < 0.001 vs. uninjured group, #P < 0.05 vs. SCI group.
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of pro-inflammatory factors and further increased the expression of the anti-inflammatory cytokine IL-10.

NF-κB has a key role in initiation of the inflammatory response, and its activation has been implicated in the pathogenesis of multiple acute and chronic inflammatory lung diseases [32, 33]. Hence, various agents that can modulate NF-κB activity have been investigated as potential therapeutic options against ALI [34, 35]. Resveratrol has also been reported to have protective effects in LPS-induced ALI [18]. Therefore, we measured the protein level of SIRT1 (a key therapeutic target of resveratrol [36]) and p-NF-κB in the lungs after SCI using western blotting. Trauma to the LTSC resulted in slightly up-regulated SIRT1 expression and significantly increased NF-κB activity in the lungs at 12 h. More importantly, resveratrol treatment induced a significant decrease in NF-κB activity, along with further increases in SIRT1 levels in the lungs after SCI. This is the first report to suggest SIRT1 to be a therapeutic target for the treatment of SCI-induced ALI.

Apoptosis has been reported to be involved in the cell loss in ALI and the subsequent histologic changes [37]. Caspase-3 is a crucial mediator of apoptosis that catalyzes the specific cleavage of many key molecules, and its activation leads to irreversible apoptosis [38]. We found that cleaved caspase-3 was over-expressed in lung tissues after SCI, and that resveratrol could suppress caspase-3 activation. Our data suggest that resveratrol might have a protective role in SCI-induced ALI through its anti-apoptosis effect.

Conclusion

We demonstrated that weight-drop trauma to LTSC induces a systemic inflammatory response and that the ensuing ALI is characterized by hemorrhage, diffuse edema and parenchymal inflammation. Resveratrol treatment can alleviate SCI-induced lung damage due to its anti-inflammatory effects. Our results suggest that resveratrol is a potential candidate for the treatment of pulmonary problems after traumatic SCI.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Tiansheng Sun, Institute of Orthopaedics, Chinese PLA Beijing Army General Hospital, Dongcheng District, 5 Nanmencang Number, Beijing 100700, China. Tel: +86 10 66721208; Fax: +86 10 84042690; E-mail: suntiansheng@163.com

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