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ORIGINAL ARTICLE

Dopamine D1 receptor agonist A-68930 inhibits NLRP3 inflammasome activation and protects rats from spinal cord injury-induced acute lung injury

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Study design: Randomized experimental study.
Objectives: The study aimed to investigate the therapeutic efficacy and molecular mechanisms of A-68930 in a rat model of spinal cord injury (SCI)–induced acute lung injury (ALI).
Setting: China.
Methods: The influences of A-68930 on the pulmonary edema, histological changes, proinflammatory cytokines levels, myeloperoxidase (MPO) activity and NLRP3 inflammasome protein expression were estimated.
Results: SCI significantly promoted NLRP3 inflammasome activation, increased proinflammatory cytokine productions and MPO activity, and induced pulmonary edema and tissue damage in the SCI group as compared with the control group. A-68930 administration significantly inhibited NLRP3 inflammasome activation and reduced inflammatory cytokines levels and MPO activity. Moreover, A-68930 administration attenuated pulmonary edema and histopathology.
Conclusion: Our experimental findings indicated that A-68930 exhibited a protective effect on SCI-induced ALI by the alleviations of inflammatory response with the inhibition NLRP3 inflammasome activation 72 h post injury. The present study indicated that A-68930 could be a potentially efficient therapeutic strategy for the treatment of SCI-induced ALI.
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INTRODUCTION

Traumatic spinal cord injury (SCI) is a disastrous event that can lead to systemic inflammatory response syndrome and secondary multiple organ complications.[1–3] Pulmonary complications are reported to be one of the major causes of death in SCI patients.[4–6] Inflammation is a crucial mechanism in the pathophysiology of SCI-induced acute lung injury (ALI).[7] Targeting of the inflammatory response can exert protective effects in SCI-induced ALI in rats.[2,7] Despite a number of trials about the relevant pathogenesis, there is a lack of effective medicine for the treatment of ALI.[8] Hence, it is urgent to explore the efficient therapeutic strategy.

Pulmonary inflammatory responses include the maturation and secretion of proinflammatory cytokines interleukin (IL)-1β and IL-18, which induce an increase in endothelial permeability.[9] The maturation and secretion of pro-IL-1β and pro-IL-18 require the activation of proteolytic enzyme caspase-1, which is mediated by the activation of nucleotide-binding domain-like receptor protein 3 (NLRP3) and subsequently the recruitment of apoptosis-associated speck-like protein (ASC).[10,11] The NLRP3 inflammasome, a kind of cytosolic protein signaling complex, consists of NLRP3, ASC and caspase-1 and is assembled after endogenous 'danger'.[12,13] The NLRP3 inflammasome regulates the maturation and release of IL-1β and IL-18;[14–16] targeting of the NLRP3 inflammasome can exert protective effects in paraquat[9] and lipopolysaccharide (LPS)[17] -induced ALI.

A-68930, a specific and a potent dopamine D1 receptor (DRD1) agonist, exhibits sedative,[18] neuroprotective, anti-stress[19] and anti-inflammatory[20] effects. DRD1 has been proved to be present in almost all immune effector cells including bone marrow-derived macrophages, microglias, astrocytes, normal human leukocytes and dendritic cells.[20–22] Dopamine suppresses NLRP3 inflammasome activation via DRD1 signaling, and DRD1 signaling induced NLRP3 ubiquitination and autophagy-mediated degradation.[20] DRD1 signaling mitigates LPS-induced systemic inflammation and monosodium urate crystal-induced peritoneal inflammation by inhibition of NLRP3 inflammasome activation.[20] Recent study has shown that A-68930 significantly controls neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neuroinflammation and reduces remarkable loss of neurons by suppressing NLRP3 inflammasome activation in mice.[20] However, no study has been performed to investigate the therapeutic efficacy and molecular mechanisms of A-68930 in SCI-induced ALI in rats.

On the basis of above considerations, we investigate whether NLRP3 inflammasome is involved in SCI-induced ALI and whether A-68930 could inhibit NLRP3 inflammasome activation with protective effects in a rat model of SCI-induced ALI.

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MATERIALS AND METHODS

Animals and groups

Adult female Sprague-Dawley rats weighing 250–300 g were purchased from Beijing Haidian Thriving Experimental Animal Centre (Beijing, China). All procedures for these experiments complied with the guidelines of the Animal Ethics Committee of Hangzhou First People's Hospital (Hangzhou, China). Rats were housed in a standard animal room with a 12 h light/dark cycle.

Eighty rats were randomly assigned into four equal groups via a random number table: (i) control group, where rats were uninjured; (ii) SCI group, where rats underwent SCI; (iii) SCI+Vehicle (Veh) group, where rats were intraperitoneally injected with 1 ml vehicle (0.1% propylene glycol in 1 ml sterile saline) immediately after SCI; and (iv) SCI+A-68930 group, where rats were intraperitoneally injected with 5 mg kg^{-1} A-68930 (Sigma–Aldrich, St Louis, MO, USA) in a 1 ml vehicle immediately after SCI. All animals in the SCI+A-68930 and SCI+Veh groups were intraperitoneally injected with an equal volume of the 5 mg kg^{-1} A-68930 or vehicle at 8-h intervals for 3 days (9 times totally). The dose and timing of A-68930 were based on previous study.[20]

Surgical procedure

SCI was induced by a model described by Young.[23] Rats were anesthetized with an intraperitoneal injection of 3.0 ml kg^{-1} 10% chloral hydrate. Laminectomy was performed to expose the spinal cord at the vertebral T9–T11 segment without damage to the dura. The spinal cord at the vertebral T10 segment (spinal T9) underwent a 25 g·cm impact. Rats were administered intramuscular injections of penicillin (400 000 unit per animal per day) and buprenorphine to prevent infection and relieve pain postoperatively. In addition, rats underwent manual bladder emptying twice a day. All rats were killed 72 h post injury on the basis of our preliminary experiment.

Pulmonary permeability index (PPI) and lung wet weight-to-dry weight (W/D) ratio

PPI and lung W/D were assessed on the basis of the method recorded by Gao *et al*.[24] 72 h after injury. The protein level in bronchoalveolar lavage fluid (BALF) and plasma was quantified by the Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL, USA). The ratio of BALF protein/plasma protein (which is regarded as the PPI) was used to assess pulmonary vascular permeability in the airway ($n=5$ in each group).

Lungs were removed, weighed and subsequently dried in an oven at 60 °C for 72 h. The purpose of lung W/D was to evaluate the extent of pulmonary edema ($n=5$ in each group).

Histological study

Seventy-two hours after injury, rats ($n=5$ in each group) were perfused with 0.9% saline and subsequently with 4% paraformaldehyde. For the histologic analyses, some paraffin lung sections were stained with hematoxylin-eosin reagent. Histologic scoring was based on (i) edema, (ii) inflammatory cell infiltration, (iii) congestion and (iv) hemorrhage, and the score of each item was recorded as follows: 0, normal; 1, mild; 2, moderate; and 3, severe.[25]

Biochemical analysis

After the BALF samples were obtained 72 h after injury, they were immediately centrifuged at 2000 r.p.m. for 15 min at 4 ° C. IL-1β, IL-18 and tumor necrosis factor (TNF)-α concentrations in the collected supernatants were determined through enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA).

Measurement of MPO activity

Myeloperoxidase (MPO), a particular oxidative enzyme, widely exists in neutrophil granules and acts as a specific marker of neutrophil infiltration and activation. The measure of MPO activity in the lung tissue was performed in the lung tissue 72 h after injury with a commercial kit (Jiancheng Co, Nanjing, China) according to the manufacturer's instructions ($n=5$ in each group).

Extraction of the protein and western blot analysis

The lung samples were removed 72 h after injury and stored at − 80 °C until use. Specimens were homogenized in RIPA buffer and then centrifuged at 12 000 r.p.m. for 30 min at 4 °C. Protein concentration in the supernatant was quantified via the Bicinchoninic Acid method. Total protein (20 μg) was separated with 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skimmed milk and subsequently incubated with specific primary antibodies overnight at 4 °C. Primary antibodies contained anti-NLRP3, anti-ASC, anti-caspase-1 and anti-β-actin (all 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with phosphate buffered saline twen (PBST), the membranes were incubated with a horseradish peroxidase-coupled secondary antibody (1:1000; Millipore). Detection of proteins was performed using the enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL, USA). Protein levels were analyzed via imaging software (Quantity One; Bio-Rad Co. Ltd., Hercules, CA, USA).

Statistical analysis

Data in the study are expressed as the mean ± s.e. of the mean (s.e.m.) and were analyzed using SPSS software version 16.0 (SPSS Inc, Chicago, IL, USA). Comparisons between different animal groups were performed by one-way analysis of variance and the Dunnett *post hoc* test. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

A-68930 administration reduces pulmonary vascular permeability and edema

The PPI and W/D ratio were used for assessing the influence of A-68930 on the pulmonary vascular permeability and edema, respectively, in SCI-induced ALI rats. Our result showed that the PPI (Figure 1a; $P<0.001$) and W/D ratio (Figure 1b; $P<0.01$) were significantly increased in the SCI group as compared with the control group. Furthermore, there was no significant difference between the

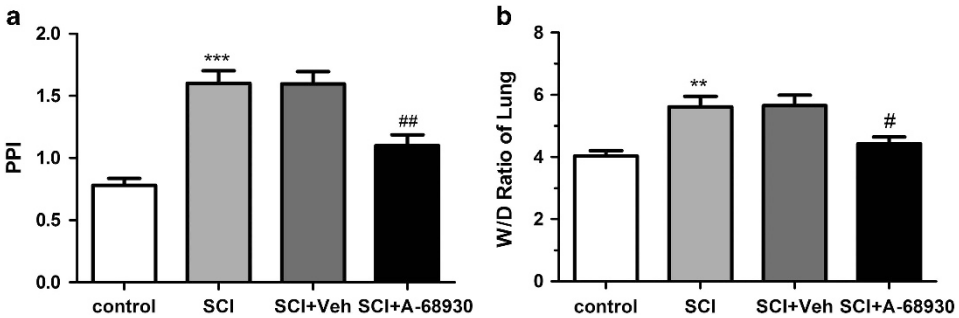

Figure 1 Effects of A-68930 on PPI (**a**) and W/D ratio (**b**) in the lung tissue 72 h after SCI. $^{**}P<0.01$ and $^{***}P<0.001$ compared with the control group; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with the SCI+Veh group. The data represent means ± s.e.m.

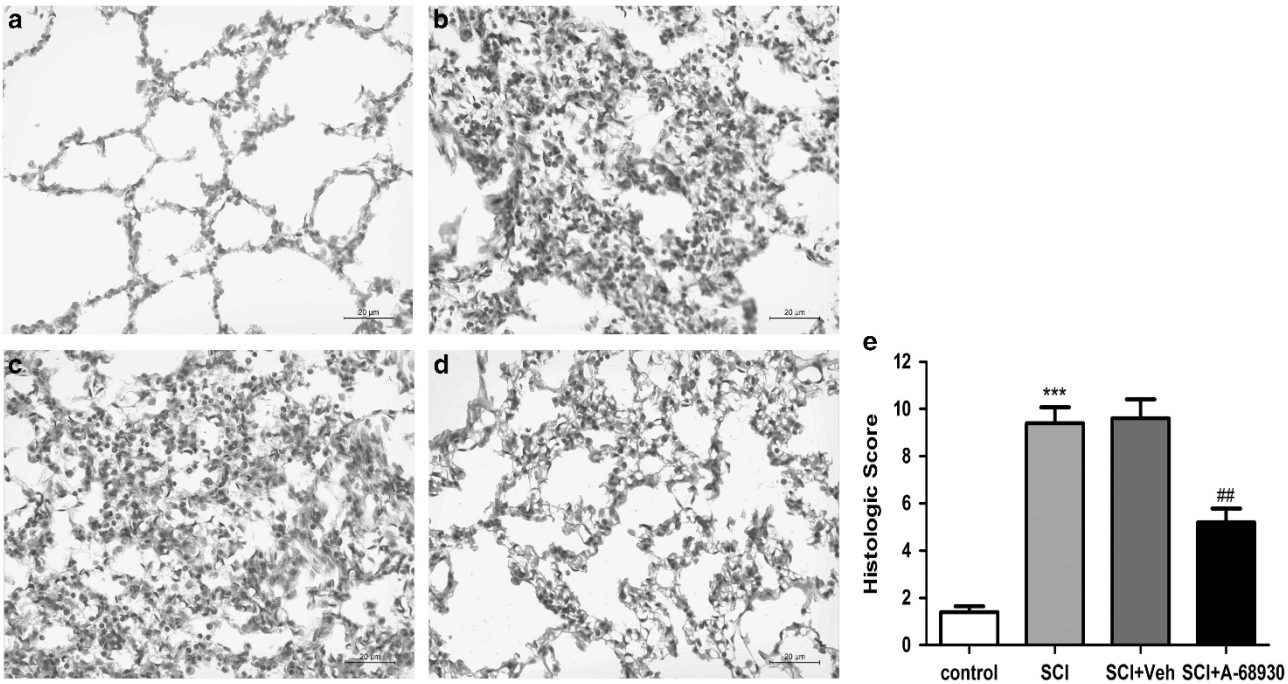

Figure 2 Effects of A-68930 on histopathologic change in the lung tissue 72 h after SCI. $^{***}P<0.001$ compared with the control group; $^{\#\#}P<0.01$ compared with the SCI+Veh group. The data represent means ± s.e.m. (**a**) control group; (**b**) SCI group; (**c**) SCI+Veh group; (**d**) SCI+A-68930; (**e**) histopathologic scores. A full color version of this figure is available at the *Spinal Cord* journal online.

SCI and the SCI+Veh groups with regard to PPI and W/D ratio. In comparison with the SCI+Veh group, rats in the SCI+A-68930 group showed a significant decrease in PPI (Figure 1a; PPI: $P<0.01$) and W/D ratio (Figure 1b; W/D ratio: $P<0.05$).

A-68930 administration reduces histopathologic damage

Histopathologic changes of the lung tissue were used for estimating the protective effects of A-68930 on rats with SCI-induced ALI. Compared with no change in the control group (Figure 2a), congestion, edema and structural disruption were shown in the SCI group (Figure 2b; $P<0.001$). Nevertheless, the changes were inhibited significantly by A-68930 administration in the SCI+A-68930 group (Figure 2d; $P<0.01$). Histopathological scores were calculated and are shown in Figure 2e.

A-68930 administration reduces proinflammatory cytokine levels

SCI significantly increased proinflammatory cytokines IL-1β, IL-18 and TNF-α productions in the SCI group (Figure 3a, IL-1β: $P<0.001$; Figure 3b, IL-18: $P<0.01$; Figure 3c, TNF-α: $P<0.001$) as compared with the control group. Furthermore, there was no significant difference between SCI and SCI+Veh groups with regard to IL-1β, IL-18 and TNF-α levels. However, A-68930 administration significantly reduced the change of proinflammatory cytokines levels in the SCI+A-68930 (Figure 3a, IL-1β: $P<0.01$; Figure 3b, IL-18: $P<0.05$; Figure 3c, TNF-α: $P<0.05$) group as compared with the SCI+Veh group.

A-68930 administration inhibits MPO activity

In comparison with the control group, the MPO activity in lung tissue (Figure 4, $P<0.05$) increased significantly in the SCI group. Furthermore, there was no significant difference between the SCI and the SCI +Veh groups. Nevertheless, A-68930 administration in the SCI +A-68930 group (Figure 4, MPO activity: $P<0.05$) group significantly inhibited MPO activity as compared with the SCI+Veh group.

A-68930 administration inhibits NLRP3 inflammasome activation

SCI induced significant upregulated protein expression of NLRP3, ASC and active-caspase-1 in the SCI group (Figure 5, NLRP3: $P<0.001$; ASC: $P<0.01$; active-caspase-1: $P<0.001$) as compared with the uninjuried group. Furthermore, there was no significant difference between the SCI group and the SCI+Veh group with regard to NLRP3, ASC and caspase-1 levels. However, A-68930 administration significantly reduced the NLRP3 and active-caspase-1 levels in the SCI+A-68930 (Figure 5, NLRP3: $P<0.01$; active-caspase-1: $P<0.01$) group as compared with the SCI+Veh group. Moreover, there was no significant difference with regard to the ASC level between the SCI+Veh group and the SCI+A-68930 group.

DISCUSSION

In the past years, A-68930, a DRD1 full agonist, is recognized as an efficient anti-stress agent.[26,27] Recent study has showed that A-68930 can exert protective effects on neurons through controlling 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neuroinflammation.[20] In the present study, we demonstrated that A-68930 administration suppressed NLRP3 inflammasome activation with a decrease in IL-1β, IL-18 and TNF-α levels, inhibition of MPO activity, reduction in pulmonary edema and vascular permeability, and alleviation of lung tissue damage in a rat model of SCI-induced ALI.

IL-1β has a pivotal role in acute lung damage,[28] which is involved in increasing other proinflammatory cytokines such as TNF-α, induction of endothelium damage and apoptosis, enhancing vascular endothelial and alveolar epithelial permeability, contributing to edema, finally leading to cell death and epithelial barrier dysfunction.[29,30] IL-1β can cause ALI through alphavbeta5 and alphavbeta6 integrin-dependent mechanisms.[31] Block of IL-1 receptor can suppress inflammation and

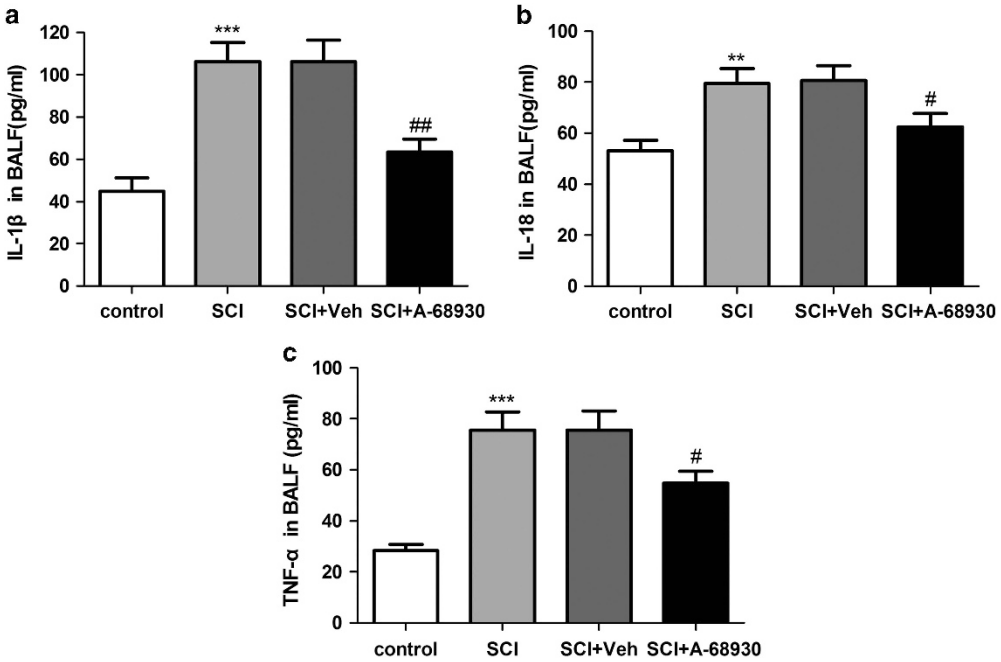

Figure 3 Effects of A-68930 on proinflammatory cytokines IL-1β (**a**), IL-18 (**b**) and TNF-α (**c**) protein levels in BALF 72 h after SCI. $^{**}P<0.01$ and $^{***}P<0.001$ compared with the control group. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with the SCI+Veh group. The data represent means ± s.e.m.

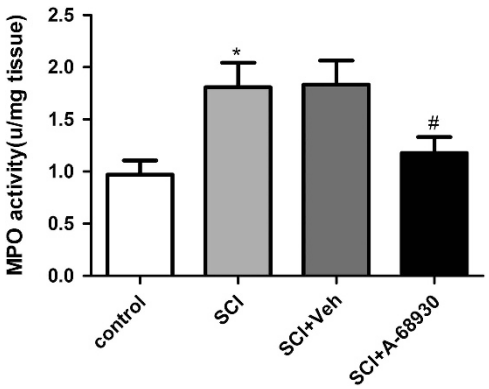

Figure 4 Effects of A-68930 on MPO activity in the lung tissue 72 h after SCI. $^{*}P<0.05$ compared with the control group. $^{\#}P<0.05$ compared with the SCI+Veh group. The data represent means ± s.e.m.

fibrosis of mesenchymal stem cells in lung injury,[32] ameliorate hypoxemia in LPS/mechanical ventilation-induced-ALI[33] and attenuate lung injury in gram-negative pneumonia.[34] IL-18 is another important proinflammatory cytokine in ALI.[35] Bleomycin-induced lung injury was markedly alleviated in IL-18Rα−/− and IL-18−/− mice.[36] TNF-α can inhibit vasodilator-stimulated phosphoprotein expression and weaken alveolar–capillary barrier function in ALI.[36] Targeting the TNF-α too can attenuate cardiopulmonary bypass-induced lung injury.[37] Anti-TNF-α antibody can alleviate inflammatory lung injury induced by cardiopulmonary bypass in a rabbit model.[38] Consistent with previous studies, we found that trauma to spinal cord causes an increase in proinflammatory cytokine levels in the BALF.[2] However, A-68930 treatment markedly downregulated the protein expressions of IL-1β, IL-18 and TNF-α. Furthermore, a decrease in the TNF-α level may be related to reduced IL-1β production, and further investigation is required.

Neutrophil influx into the lung tissue with increasing oxidative and proteolytic enzymes levels can induce endothelial cell damage and overwhelming inflammation.[39] The intensity of neutrophil influx is related to the severity of lung injury in acute respiratory distress syndrome patients.[40] Targeting neutrophil exocytosis can alleviate ALI in a rat model of immune complex deposition.[41] Consistent with previous studies,[2] trauma to spinal cord caused an increase in neutrophil infiltration and activation in lung tissue. However, our study showed that the increase in MPO activity was markedly inhibited by A-68930 administration.

Importantly, the NLRP3 inflammasome is increasingly recognized as an important proinflammatory mediator regulating the maturation and release of IL-1β and IL-18.[20] Most recently, the NLRP3 inflammasome has been reported to have an important role in paraquat-,[9] LPS-[33] and mechanical ventilation[42]-induced ALI, and targeting of the NLRP3 inflammasome can inhibit inflammation and alleviate pulmonary edema and tissue damage in LPS-induced ALI rats.[17] Silencing of NLRP3 inflammasome inhibits ceramide-induced alveolar epithelial permeability and proinflammatory cytokine secretion.[29] NLRP3 deletion can suppress pulmonary inflammation and epithelial cell apoptosis in hyperoxia-induced ALI mice.[43] Moreover, inhibition of NLRP3 inflammasome attenuated histopathologic changes, MPO activity and protein concentrations in the BALF in burn-induced ALI.[44] In this study, we found that SCI caused NLRP3 inflammasome activation in the lung tissue, and A-68930 administration significantly inhibited the inflammasome activation.

CONCLUSION

In conclusion, our experimental findings indicated that A-68930 exhibited a protective effect on SCI-induced ALI by the alleviations of inflammatory response with the inhibition NLRP3 inflammasome activation 72 h post injury. The present study indicated that A-68930 could be a potentially efficient therapeutic strategy for the treatment of SCI-induced ALI.

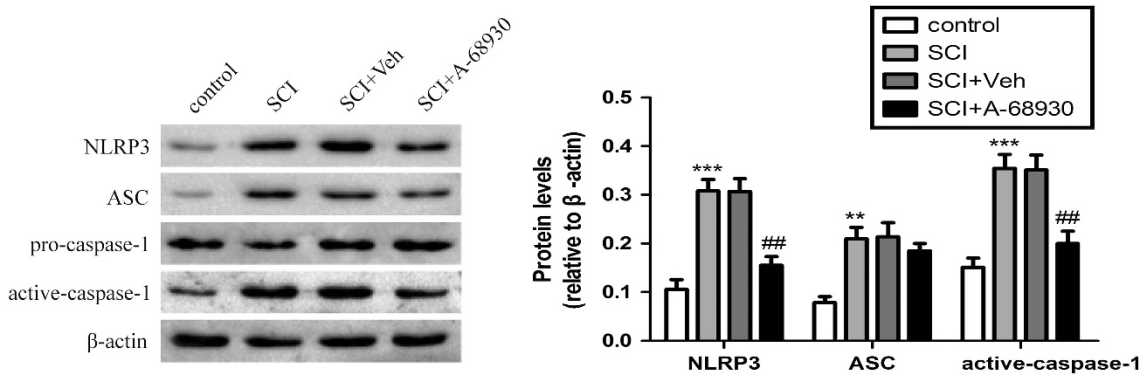

Figure 5 Effects of A-68930 on NLRP3, ASC and active-caspase-1 protein levels in the lung tissue 72 h after SCI. $^{**}P<0.01$ and $^{***}P<0.001$ compared with the control group. $^{\#\#}P<0.01$ compared with the SCI+Veh group. The data represent means ± s.e.m.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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