Department of Pain Management[1], Shandong Provincial Hospital, Shandong University, Ji'nan; Department of Anesthesiology[2], Weifang Yidu Central Hospital; Department of Anesthesiology[3], Qingzhou People's Hospital, Qingzhou, P.R China

Histone deacetylase inhibitors trichostatin A and suberoylanilide hydroxamic acid attenuate ventilator-induced lung injury

Hua-Yong Chen[1,2], Li Li[3], Zhi-Jian Fu[1]

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Zhi-Jian Fu, Department of Pain Management, Shandong Provincial Hospital, Shandong University, 324#, Jingwu Road, Ji'nan, 250012, P.R China
chuayong0411@163.com

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The pathophysiology of ventilator-induced lung injury (VILI) involves multiple mechanisms including inflammation. Histone deacetylase inhibitors have been shown to exert anti-inflammation activity. The purpose of this study was to examine the protecting roles and mechanisms of the histone deacetylase inhibitors trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) in ventilator-induced lung injury in normal rat lung. Male Sprague-Dawley rats were divided into four groups: lung-protective ventilation (LV), injurious ventilation (HV), HV+TSA and HV+ SAHA groups. Mechanical ventilation (MV) settings were 7 ml/kg VT and 3 cm H_2O positive end-expiratorypressure [PEEP], 40 breaths/min for LV group and 42 ml/kg VT, zero end-expiratoryvolume [ZEEP], 40 breaths/min for the HV, HV+TSA and HV+ SAHA groups. After 2 h of MV, acute lung injury (ALI) score, wet-to-dry (W/D) weight ratio and the activity of myeloperoxidase (MPO) were determined. The concentration of tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-10(IL-6) in the homogenized lung were measured by ELISA. The expression ICAM-1 was measured by both realtime PCR and Western blot assays. In addition, survival of each group was also assessed. Our results indicated that administration of TSA or SAHA alleviated ventilator-induced lung injury. This was accompanied by reduced neutrophil infiltration, reduced MPO activity, decreased intercellular adhesion molecule-1 (ICAM-1) expression in lung tissue, and lower TNF-α, IL-1β and IL-6 levels. In addition, treatment with HDAC inhibitors significantly prolonged the survival time of ventilator-induced lung injury rats. Our data suggested that TSA and SAHA could significantly alleviate ventilator-induced rat lung injury and prolong the survival time of those rats by attenuate intrapulmonary inflammatory response.

1. Introduction

Despite recent advances in supportive care, a high mortality rate still persists in acute respiratory failure patients. The use of mechanical ventilation (MV) is well established in intensive care medicine and is a life-saving therapy in patients with acute respiratory failure. The use of lower tidal volumes (VT) significantly reduced morbidity and mortality in acute respiratory failure patients (Kuipers et al. 2011). However, it is well accepted that MV can aggravate and even induce lung injury in the healthy lung, termed ventilator-induced lung injury (VILI) (Gajic et al. 2004). Recent studies indicate that proinflammatory cytokines play an important role in the development of VILI (Ricard et al. 2001). In the development of VILI, injured regions are fluid filled or collapsed, while healthy regions remain well aerated and are at risk for overdistention. Repetitive opening and closure of collapsed lung parts induces shear forces acting on pulmonary cells causing atelectrauma, which could cause gene expression changing, and induce the release of inflammatory cytokines and chemokines (Muscedere et al. 2007).

The expression of various inflammation-related genes is tightly regulated at multiple checkpoints. Histone acetylation and deacetylation regulate the structure and function of chromatin and therefore regulate downstream genes expression at transcriptional level (Ito 2007). Histone lysine acetylation generally induces transcriptional activation by way of neutralization of the positive charges on lysines and subsequent chromatin relaxation, which is associated with the opened structure of chromatin, accessibility to transcription factors, and eventually transcriptional activation (Berger 2002). The dynamic equilibrium in the acetylation level of lysine residues in histones is maintaining by two groups of functional opposite enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC) (Yang and Seto 2007).

Inhibition of HDAC is emerging as a novel approach to treat a variety of diseases including various cancers (Pan et al. 2007). As HDAC inhibition prompts tumor cells to enter apoptosis, small-molecule HDAC inhibitors have been developed as a new class of mechanism-based anti-cancer agent, many of which have entered clinical trials (Mehnert and Kelly 2007). Recently, broad acting inhibitors of HDAC have been shown to have anti-inflammatory effects both *in vitro* and *in vivo* (Leoni et al. 2005). HDAC inhibitors significantly attenuated inflammatory injury in airway, digestive tract, and joints in several animal models (Choi et al. 2005; Glauben et al. 2006; Lin et al. 2007; Han and Lee 2009). It is significant that these anti-inflammatory

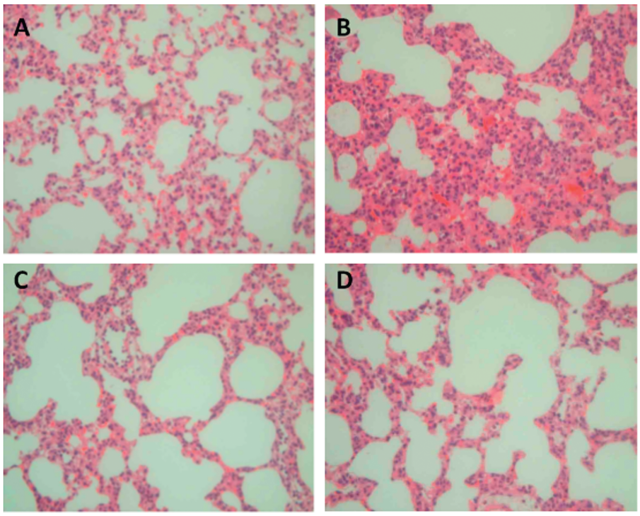

Fig. 1: Treatment with TSA or SAHA alleviated ventilator-induced morphologic lesions in lung tissue. Histopathological changes in the lung. Lung samples harvested after mechanical ventilation were processed and stained with hematoxylin and eosin (HE) for morphological evaluation. (A) LV group. (B) HV group. (C) HV+TSA group. (D) HV+SAHA group. Representative lung sections of each group are shown.

effects are observed at 10–100 fold lower concentrations than their anti-cancer effects (Cantley and Haynes 2013).

Recent studies have demonstrated that HDAC inhibitors effectively attenuated LPS-induced inflammation and attenuate acute lung injury during cecal ligation and puncture-induced polymicrobial sepsis (Zhang et al. 2010). However, whether histone deacetylase inhibitors could inhibit ventilator-induced lung injury remains to be elucidated. The purpose of this study was to examine the protective roles and mechanisms of histone deacetylase inhibitors trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) in VILI in normal rat lung.

2. Investigations and results

2.1. Treatment with TSA or SAHA alleviated ventilator-induced morphologic lesions in lung tissue

As shown in Fig. 1A, there were no obvious morphological changes in lung tissue from the LV group. However, lung specimens from the HV group with vehicle-treated animals displayed significant histological abnormalities, including infiltration of leukocytes into the interstitial spaces, hemorrhage, and marked swelling of the alveolar walls (Fig. 1B). TSA or SAHA pretreatment significantly attenuated these histological alterations (Fig. 1C and 1D). These histological observations suggested that histone deacetylase inhibitors could alleviate ventilator-induced morphologic lesions in lung tissue.

2.2. Treatment with TSA or SAHA reduced lung edema caused by ventilator-induced lung injury

One of the typical features of ventilator-induced lung injury is edema. Indeed, in the vehicle-treated, HV group a marked increase in lung water content was immediately observed when compared with the LV group (expressed by wet weight/dry weight [W/D] ratio) (Fig. 2). Pretreatment with TSA or SAHA could significantly reduce the W/D weight ratio compared with the HV group (Fig. 2). These data suggested that histone deacetylase inhibitors could reduce lung edema caused by ventilator-induced lung injury.

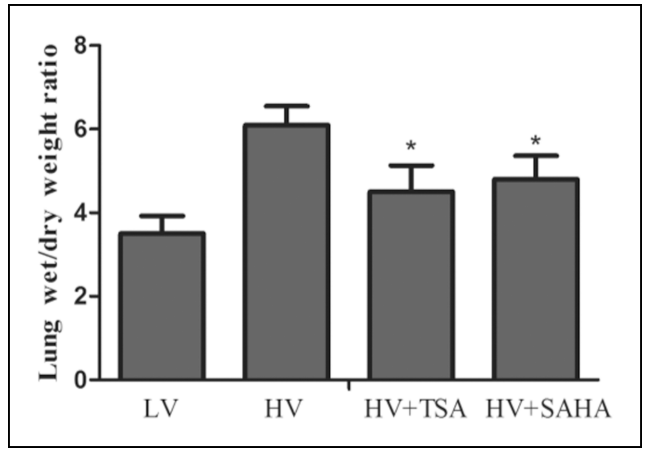

Fig. 2: Treatment with TSA or SAHA reduced lung edema caused by ventilator-induced lung injury. The Wet/dry weight ration of lung tissues from rats with indicated treatment was measured. Data are expressed as mean ± SEM of the values of 5 rat of each group. * $p < 0.05$ compared with the HV group.

2.3. Treatment with TSA or SAHA reduced leukocyte infiltration caused by ventilator-induced lung injury

Moreover, the infiltration of neutrophils into the lung tissue was also determined by measuring the activities of MPO, a reliable marker of neutrophil infiltration. In the HV group, the activity of MPO was obviously higher than in the LV group (Fig. 3). However, administration of the HDAC inhibitor TSA significantly reduced the MPO activities in lung compared with the HV group (Fig. 3). Similar results were obtained in the HV+ SAHA group (Fig. 3). Taken together, these data suggested that histone deacetylase inhibitors could reduce lung edema and leukocyte infiltration caused by ventilator-induced lung injury.

2.4. Treatment with TSA or SAHA down-regulated the expression of ICAM-1

ICAM-1 is known to be involved in intrapulmonary recruitment of leukocytes. We proposed that ICAM-1 expression would be up-regulated in the HV group. Indeed, compared with the control group, ICAM-1 mRNA expression (Fig. 4A) was significantly elevated in the HV group. TSA or SAHA also markedly decreased ICAM-1 protein levels (Fig. 4B).

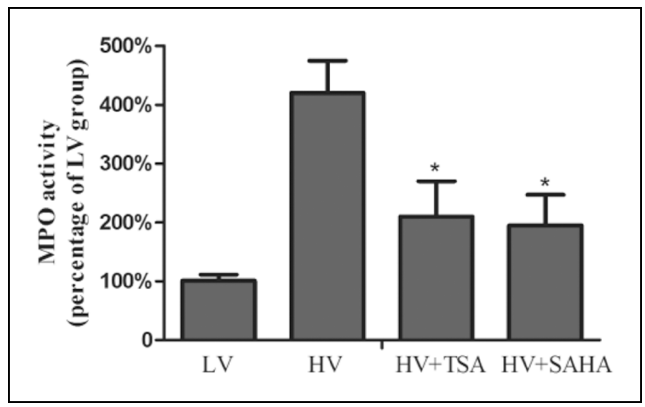

Fig. 3: Treatment with TSA or SAHA reduced leukocyte infiltration caused by ventilator-induced lung injury. The myeloperoxidase (MPO) activity in lung tissues from rats with indicated treatment was measured. Data are expressed as mean ± SEM of the values of 5 rat of each group. * $p < 0.05$ compared with the HV group.

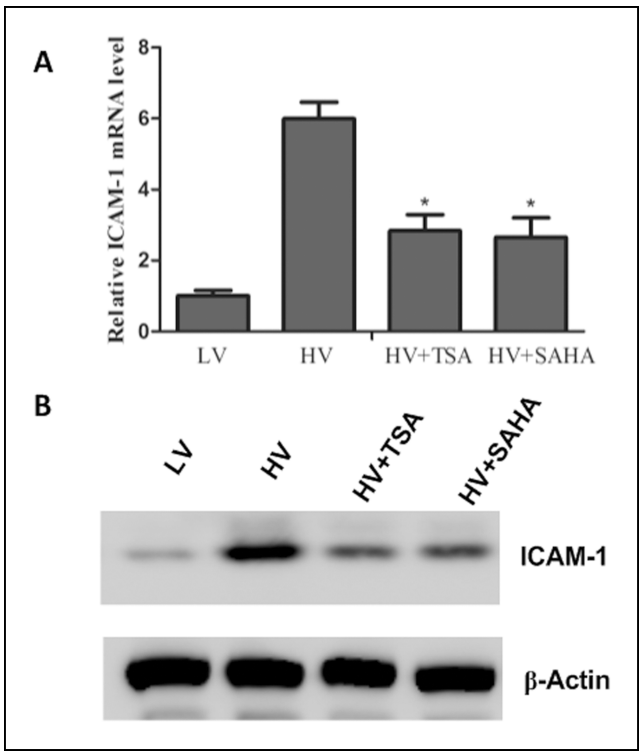

Fig. 4: Treatment with TSA or SAHA downregulated the expression of ICAM-1. (A) The mRNA levels of ICAM-1in lung tissues from rats with indicated treatment was measured by Realtime PCR. (B)The protein levels of ICAM-1in lung tissues from rats with indicated treatment was measured by Western blot with ICAM-1and β-Actin antibodies.

2.5. Treatment with TSA or SAHA suppress TNF-α, IL-1β, and IL-6 elevation caused by ventilator-induced lung injury

Activation of inflammatory mediators, including TNF-α, IL-1β, and IL-6in the homogenized lung, is considered to play a major role in the pathogenesis of lung injury. Indeed, ventilator-induced lung injury was associated with marked increases in the protein levels of TNF-α (Fig. 5A), IL-1β (Fig. 5B), and IL-6 (Fig. 5C). Administration of either TSA or SAHA significantly suppressed TNF-α, IL-1β, and IL-6 elevation.

2.6. Treatment with TSA and SAHA delayed the death of rats with ventilator-induced lung injury

To determine the effect of HDAC inhibitors on the outcome of ventilator-induced lung injury, the survival rate between vehicle and TSA- or SAHA -treated rats was compared. Kaplan–Meier survival curves (Fig. 6) showed that treatment with TSA or SAHA significantly prolonged the survival time of rat with ventilator-induced lung injury when compared with vehicle group.

3. Discussion

Activation of inflammatory mediators is considered to play a major role in the pathogenesis of injurious ventilation (Bhatia and Moochhala 2004). TNF-α and IL-1 shared many of the same effects, including neutrophil recruitment, stimulation of chemokine release, and up-regulation of adhesion molecules. Several studies suggest that IL-6 plays a role in the development of distal organ failure in ventilator-induced lung injury (Anonymous 2000; Ranieri et al. 2000). The administration of HDAC inhibitors usually results in enhanced acetylation of histone. This alteration might be associated with the open

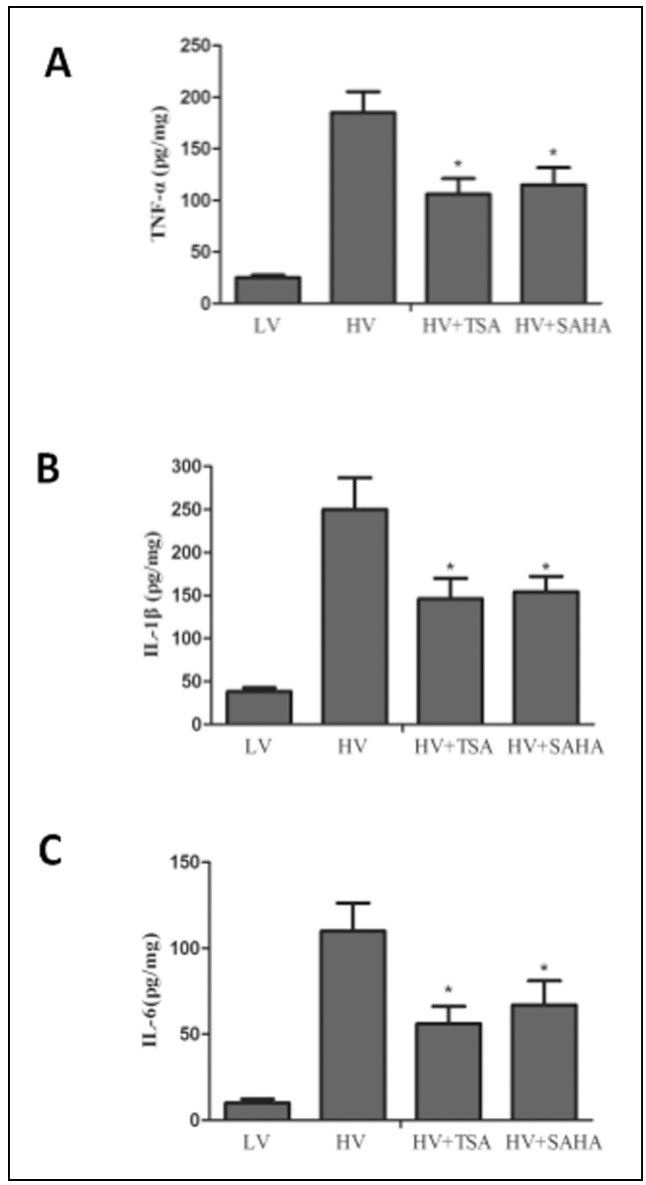

Fig. 5: Treatment with TSA or SAHA suppress TNF-α, IL-1β, and IL-6 elevation caused by ventilator-induced lung injury. (A) The concentration of TNF-α in plasma of rats with indicated treatment was monitored by ELISA. (B) The concentration of IL-1β in plasma of rats with indicated treatment was monitored by ELISA. (C) The concentration of IL-6 in plasma of rats with indicated treatment was monitored by ELISA. * $p < 0.05$ compared with the HV group.

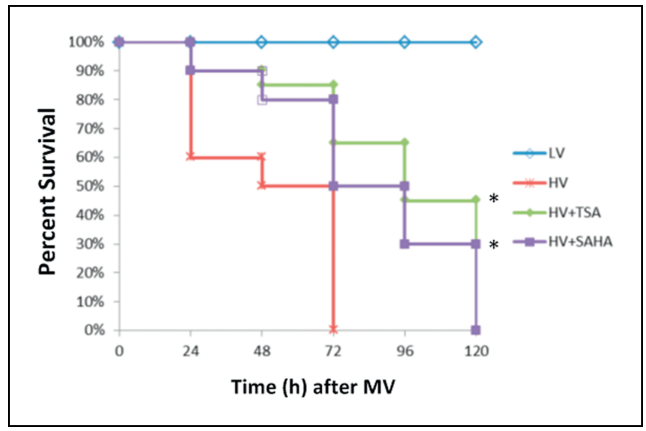

Fig. 6: Treatment with TSA and SAHA delayed the death of rats with ventilator-induced lung injury. The Kaplan–Meier survival curves of rats (n = 10) with indicated treatment were monitored. * $p < 0.05$ compared with the HV group.

structure of chromatin and facilitate transcription. Furthermore, ICAM-1 has been reported to be required for neutrophil recruitment during inflammatory response (Lauterbach et al. 2008). These results seem to parallel our observations that inflammatory mediators were significantly increased in a rat model of ventilator-induced lung injury. It has been reported that HDAC inhibitor could increase the transcription of TNF-α, but reduce the protein level of TNF-α in the hemorrhaged leukocytes after LPS treatment (Sailhamer et al. 2008). Therefore, the anti-inflammatory property of HDAC inhibitors seems inconsistent with their capacity to alter the chromatin structure and facilitate transcription. In agreement with this, our observation showed that HDAC inhibitors TSA and SAHA significantly suppressed TNF-α, IL-1β, and IL-6 elevation during injurious ventilation. Neutrophil rolling along the endothelium may initiate a cascade of cellular interactions, resulting in endothelial damage and subsequent development of multiple organ damage (Grommes and Soehnlein 2011). Our data showed that MPO activity, a marker for neutrophil influx, was greatly enhanced. Moreover, neutrophil infiltration was directly displayed in histological observation. HDAC inhibitors TSA and SAHA not only decrease the activity of MPO but also alleviated ventilator-induced morphologic lesions in lung tissue and prolonged the survival time of ventilator-induced lung injury rats. These results showed an activated inflammatory response during injurious ventilation and TSA and SAHA could alleviate inflammatory and largely prevent ventilator-induced lung injury.

In conclusion, our study described the anti-inflammatory effect of HDAC inhibitors during injurious ventilation. TSA and SAHA, two structurally unrelated HDAC inhibitors, significantly alleviated ventilator-induced lung injury and prolonged the survival time of injurious ventilation treated rats. Our results suggest that the administration of HDAC inhibitors seems a promising anti-inflammatory strategy to regulate the expression of inflammatory genes and control ventilator-induced lung injury.

4. Experimental

4.1. Animals

Specific pathogen-free male Sprague-Dawley rats weighing 240–290 g were obtained from the Shandong Animal Centre (Shangdong, China). Rats were housed in pathogen-free laboratory for 72 h with free access to water and food. The experimental protocol was approved by the Animal Care and Scientific Committee of Weifang Yidu Central Hospital Qingzhou, P.R China.

4.2. Mechanical ventilation

Rats received TSA (2 mg/kg, dissolved in phosphate buffered saline [PBS] containing 1% DMSO) or SAHA (200 mg/kg) or an equal volume of vehicle (PBS containing 1% DMSO) intraperitoneally. Thirty minutes later animals were anesthetized by intraperitoneal injection of thiopental (37 mg/kg) and then orally intubated, mechanically ventilated as reported previously. To maintain anesthesia each animal was injected with succinylcholine (5 mg/kg) *via* the external jugular vein, after which the animal was ventilated with a rodent volume ventilator. Two ventilation modalities were used, for 2 h each, as follows: (1) Rats received vehicle, with low V T ventilation (7 ml/kg VT and 3 cm H_2O positive end-expiratorypressure [PEEP], 40 breaths/min) (LV group) and (2) an injurious strategy, using a high VT and no PEEP (42 ml/kg VT, zero end-expiratoryvolume [ZEEP], 40 breaths/min) (HV group, HV+TSA group and HV+ SAHA group) (Karzai et al. 2005). The rats were killed by an intravenous injection of thiopental at the end of the mechanical ventilation period, the thorax was opened, and blood was sampled by cardiac puncture. Simultaneously, three BAL procedures were performed, each with 2 ml of normal saline. The retrieved fluid and the blood were centrifuged (2,000 g, for 10 min), and the supernatant and plasma were stored for further processing. To determine the effect of TSA or SAHA on mortality from ventilator-induced sepsis, survival after mechanical ventilation was assessed and the cumulative survival curve was depicted using the Kaplan–Meier method.

4.3. Histopathologic examination

For each lobe, a sample measuring around 1 cm^3 focused on a macroscopic lesion was excised for microscopic examination. Samples were fixed by immersion in 4% paraformaldehyde, dehydrated with a graded alcohol series, and embedded in paraffin. Paraffin-embedded sections were stained with hematoxylin and eosin.

4.4. Pulmonary capillary leakage

Pulmonary capillary leakage was determined with the Evans blue dye extravasation method. 2% Evans blue (20 mg/kg; Sigma Chemical CO) was injected IV via the tail vein 15 min before death. The lung tissues were excised and weighed. To each sample of tissues, 4.0 mL formamide was added and incubated at 37 °C for 24 h. After filtration with a glass filter, the absorbance of the filtrate was measured at 620 nm in a spectrophotometer (Beckman Instruments, Fullerton, CA). The total amount of dye could be calculated by means of a standard calibration curve. Microvascular permeability in the lungs was shown as the μg of Evans blue in every mg of tissue.

4.5. Production of inflammatory cytokines in the lungs

The levels of inflammatory cytokines in lung homogenates were quantified using enzyme-linked immunosorbent assay (ELISA) kits specific for various rat cytokines according to the manufacturer's instructions (TNF-α from Diaclone Research, France; IL-1β, and IL-6 from Biosource Europe SA, Belgium). Results were expressed as pg/mg protein.

4.6. Pulmonary myeloperoxidase (MPO) activity

Lung tissue was homogenized for 30 s in 4 ml of potassium phosphate buffer (20 mmol/L, pH 7.4) and centrifuged for 30 min at 40,000 g and 4 °C. The pellet was resuspended in 4 ml of potassium phosphate buffer (50 mmol/L, pH 6.0) containing 0.5 g/dL of cetrimonium bromide. Resuspended pellets were frozen at –70 °C until the MPO assay was performed. Frozen samples were thawed, sonicated for 90 s, incubated in a 60 °C water bath for 2 h, and centrifuged for 10 min at maximum speed. Then 0.1 ml of supernatant was added to 2.9 ml of potassium phosphate buffer (50 mmol/L, pH 6.0) containing 0.167 mg/ml o-dianisidine and hydrogen peroxide. The absorbance change at 460 nm was monitored for 3 min with a spectrophotometer.

4.7. RNA isolation and real-time PCR

Total RNAs were isolated from Rat lung tissues by TRIzol reagent, and reverse transcriptions were performed by Takara RNA PCR kit (Takara, China) following the manufacturer's instructions. In order to quantify the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) on Light Cycler 480 (Roche, Switzerland). The primer sequences used are available upon request.

4.8. Western blot

Lungs were lysed in Laemmli buffer (100 mM Tris-HCl at pH 6.8, 200 mM DTT, 4% SDS [w/v], 20% glycerol), denatured for 10 min at 80 °C, sheared with a syringe, and resolved on SDS/PAGE gels. After immunoblotting, the membranes were blocked in PBS/0.1% Tween-20 with 5% nonfat dry milk. The membranes were then incubated with primary antibodies followed by HRP-linked secondary antibodies. The signals were detected by a SuperSignal West Pico Chemiluminescent Substrate kit according to manufacturer's instructions. Antibodies directed against ICAM-1 and β-actin were purchased from Santa Cruz Biotechnology (USA).

4.9. Statistical analysis

All data were analyzed using SPSS version 14 software (SPSS Inc., Chicago, IL). All data are presented as mean ± SEM. Statistical differences were determined by a two-tailed t test. Survival statistics were compared with a Kaplan–Meier curve and log-rank test. A difference with $P < 0.05$ was considered statistically significant.

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