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Sirt 1 activator attenuates the bleomycin-induced lung fibrosis in mice *via* inhibiting epithelial-to-mesenchymal transition (EMT)

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of resveratrol on the idiopathic bleomycin (BLM)-induced pulmonary fibrosis, which is increasingly recognized as an epithelial-to-mesenchymal transition (EMT)-associated disease.

MATERIALS AND METHODS: We evaluated the effect of resveratrol on the BLM-induced fibrosis in a mouse model, via monitoring the pathological chance in mice lung, the mice body weight change and the mice death. And we also explored the regulation by BLM on (and) resveratrol on the expression and activity of Sirt 1 and on the expression of epithelial-to-mesenchymal transition (EMT)-associated markers in mice lung.

RESULTS: It was demonstrated that resveratrol ameliorated the BLM-induced fibrosis-like pathological change in mice lung, inhibited BLM-induced mice body weight loss and death. Moreover, resveratrol also inhibited the BLM-induced EMT-associated molecular events, such as reduced E-cadherin and elevated collagen I and α-SMA. We also confirmed the amelioration by resveratrol on the BLM-mediated inhibition of Sirt 1 in expression and activity in mice lung.

CONCLUSIONS: Our study confirmed the inhibitory role of resveratrol in the BLM-induced pulmonary fibrosis in a mouse model. Resveratrol ameliorated the BLM-induced pathological change of fibrosis, mice body weight loss and death. And such amelioration might be associated with the activation of Sirt 1 in mice lung. The present study implied that resveratrol might be a promising agent for effective control the pulmonary fibrosis.

Key Words:
Resveratrol, Bleomycin (BLM), Pulmonary fibrosis, Epithelial-to-mesenchymal transition (EMT), Sirt 1.

Introduction

It is currently believed that pulmonary fibrosis, particularly idiopathic pulmonary fibrosis (IPF), is still a fatal disease in adult humans. And it is hypothesized as unknown environmental and/or occupational factors or an epithelial-fibroblastic disease, which is caused by such risk factors as smoking, polluted air, gastroesophageal reflux disease, genetic factors[1,2] and commonly prescribed drugs, such as bleomycin (BLM)[3]. Transforming growth factor-β (TGF-β)/Smad signaling has been indicated as the final common pathway causing pulmonary fibrosis[4-6]. Silent information regulator 1 (Sirt 1), also known as nicotinamide adenine dinucleotide (NAD)-dependent class III histone deacetylase[7], has been confirmed to play a key role in metabolism and acute stress resistance[8-11]. Furthermore, Sirt 1 is known to inhibit the transcriptional activity of NF-κB[12], hence affecting many of its downstream mediators. Noteworthy, the Sirt 1 activator, resveratrol, has been recognized to present anti-inflammatory and antifibrotic effects in the lung[13].

Idiopathic pulmonary fibrosis is currently conceived as an epithelial-fibroblastic disease. BLM has been indicated to pose lung toxicity during the treatment of such malignancy as lymphoma[14] and squamous cell carcinomas[15]. And there were approximately 1% patients suffering from pulmonary fibrosis post receiving BLM treatment[16]. And the studies in animal models also find the BLM-induced experimental lung fibrosis[16,17], which is characterized by inflammatory and fibrotic reactions within a short period of time, even more so after intratracheal instillation. However, little is known about the role of Sirt 1 in the BLM-induced pulmonary fibrosis.

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In the present study, we evaluated the therapeutic role of resveratrol in the BLM-induced fibrosis in a mouse model, via monitoring the pathological chance in mice lung, mice body weight change and death. And we also explored the regulation by BLM (and) resveratrol on the expression and activity of Sirt 1 and the expression of epithelial-to-mesenchymal transition (EMT)-associated markers in mice lung. Our study indicated the protective role of resveratrol in the BLM-induced mice pulmonary fibrosis via promoting Sirt 1 activity and via inhibiting EMT.

Materials and Methods

Reagents and mouse model

Bleomycin and resveratrol were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and were dissolved in physiological saline. 6 to 8-week-old male Balb/c mice (average body weight of 15.103 ± 0.571 g, 25 mice in each group) were intratracheally injected with 50 μl per mouse physiological saline (Normal control), with 50 μl per mouse physiological saline containing 100 μg bleomycin (bleomycin group) or (and) with 50 μg resveratrol per mouse (bleomycin + RSV group), under anesthesia with ketamine (100 mg/kg)-xylazine (10 mg/kg). The body weight and survival (N = 10 in each group) of mice were monitored daily. And 5 mice in each group (control group, bleomycin (BLM) group and bleomycin + RSV group) were sacrificed at 10 day post-treatment, and the mouse lung was processed for routine paraffin embedding, and serial sections (5 μm) were stained with hematoxylin and eosin. Another 10 mice in each group were sacrificed at 5 or 10 day post-treatment (5 mice in each time point) for the mRNA isolation or protein isolation. All animal studies, including the conditions described above, were carried out under the approval by the Institute Animal Care and Use Committee in our Hospital.

mRNA preparation and quantitative analysis

The entire mouse lung was removed and homogenized in 5× volume ice-cold sterilized PBS (5 μl PBS for 1 μg lung tissues). And mRNA samples from lung tissues were prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the product's manual. Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed to quantify the mRNA levels of Sirt 1 and Sirt 2, with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control. One step RT-PCR Kit (Takara, Tokyo, Japan) according to the kit's protocol. The primers for Sirt 1, Sirt 2 of for GAPDH were synthesized by Sangon Biotech (Sangon, Shanghai, China). The mRNA level for Sirt 1 or Sirt 2 was normalized to GAPDH, and was presented as the fold change over control with the $^{\Delta\Delta}$Ct method[18].

Cellular protein preparation and western blot analysis

Protein Extraction Kit (Qiagen, GmbH, Hilden, Germany) was adopted to isolate proteins from mice lung under the guidance of kit's manual. And each protein sample was supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein samples were subject to the electrophoresis with 12% SDS-PAGE gel and to nitrocellulose membrane (Millipore, Bedford, MA, USA) transfer. Then, the membrane was blocked with 2% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) overnight at 4 °C, was inoculated with the rabbit anti-mouse Sirt 1 (ab12193, Abcam, Cambridge, UK), Sirt 2 (ab12749, Abcam, Cambridge, UK), GAPDH (G9545, Sigma-Aldrich, St. Louis, MO, USA), E-cadherin (ab15148, Abcam, Cambridge, UK), collagen I (ab34710, Abcam, Cambridge, UK), or with mouse anti-mouse α-Smooth Muscle Actin (α-SMA) (sc-53142, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. The specific binding of antigen and antibody was presented with the incubation with the peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) and the electrochemiluminescence (ECL) detection system (Thermo Scientific, Rockford, IL, USA). The protein level was presented as a ratio to GAPDH.

Statistical Analysis

Statistical analyses were performed with GraphPad software 6 (GraphPad Software, La Jolla, CA, USA). Results were presented as mean ± SD, and the difference between two groups was analyzed by a two-way ANOVA method or unpaired Student's *t*-test. A p value less than 0.05 was considered to be statistically significant.

Results

Resveratrol ameliorates the BLM-induced fibrosis in mouse model

To construct a BLM-induced pulmonary fibrosis in a mouse model, we observed the pathologi-

cal change in the lung of mice which were injected intratracheally with BLM. Mice after BLM treatment at 5 or 10 days were sacrificed, and the mice lung tissues were collected for hematoxylin and eosin stain (H&E stain) and were observed under optical microscope. It was indicated in Figure 1 that compared to the normal mouse lung (Figure 1A), there were a significant accumulation of inflammatory cells, collagen content, fibrosis-like change and collapsed alveolar spaces in the lung of BLM-treated mouse (Figure 1B). However, such fibrosis-like change could be ameliorated by the administration of resveratrol (Figure 1C).

Resveratrol ameliorates the body weight loss and death of BLM-treated mice

The body weight and death of mice which were treated with physiological saline (Normal control), BLM or the combination of BLM and resveratrol were monitored daily. As shown in Figure 2A, the body weight of normal mice increased from 15.315 ± 0.541 g at 0 day post-treatment, to 19.416 ± 1.633 g at 15 day post-treatment (N = 10), while the body weight of the BLM-treated mice decreased from 14.894 ± 0.582 g at 0 day post-treatment, to 10.380 ± 1.306 g at 15 day post-treatment (N = 10), with a significant difference between the two groups ($p < 0.001$). However, the body weight of the mice with the treatment with both BLM and resveratrol decreased from 15.100 ± 0.574 g at 0 day post-treatment, decreased to 14.060 ± 1.133 g at 10 day post-treatment, and then reversed to 15.340 ± 1.507 g at 15 day post-treatment, with a marked less body weight loss than the BLM-treated mice ($p < 0.01$). However, the mice body weight in this group was still significantly less than in the normal group ($p < 0.01$). Moreover, there was also difference in the mice survival among the three groups. There was (were) 1 dead mouse and another 2 in the BLM group at 8 and 11 day post-treatment respectively; whereas only 1 mouse died in the BLM & resveratrol group at 12 day post-treatment and no mouse died in the control group. Taken together, the resveratrol ameliorates the body weight loss and death of BLM-treated mice.

Resveratrol inhibits the BLM-mediated Sirt 1 downregulation in mice lung

To investigate the influence of the treatment with BLM or (and) resveratrol on the Sirt 1 activity in mice, we then examined the expression and activity of Sirt 1 in the mice in each group. Real-time quantitative PCR analysis indicated

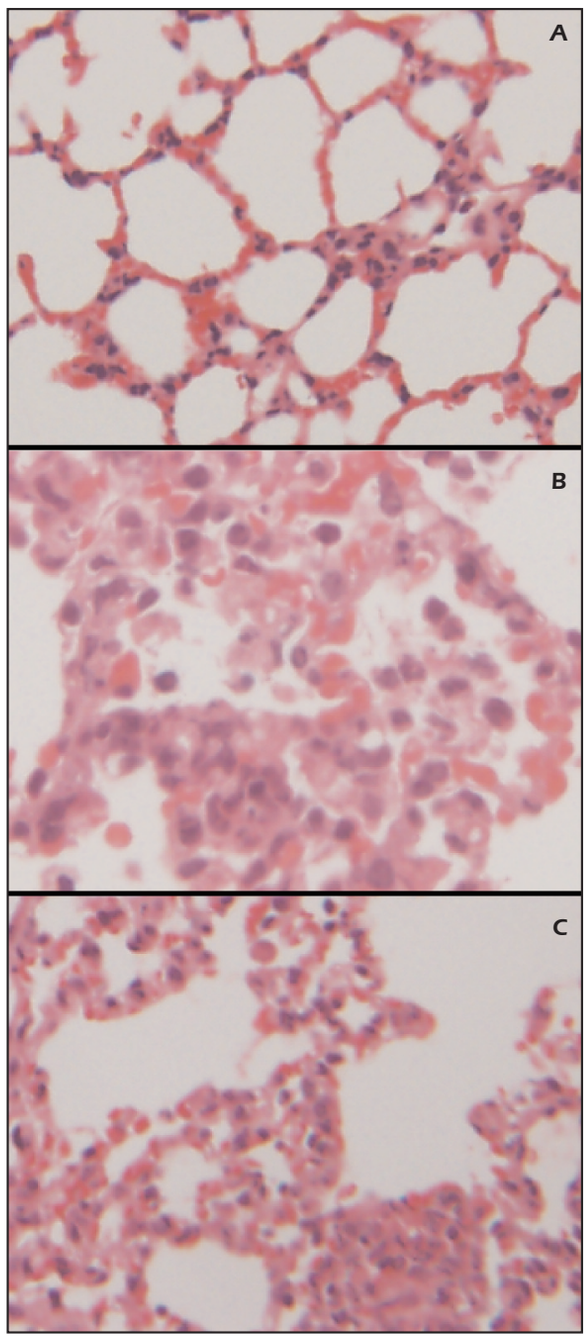

Figure 1. Pathological change in the lung of mice, treated with bleomycin or (and) resveratrol. Mice (average body weight of 15.103 ± 0.571 g, N = 5 in each group) were intratracheally injected with 50 μl per mouse physiological saline (normal control), with 100 μg bleomycin per mouse (dissolved in 50 μl physiological saline) (bleomycin group) or with both 100 μg bleomycin and 50 μg resveratrol per mouse (dissolved in 50 μl physiological saline) (bleomycin + RSV group) at 0 day. Mice were sacrificed at 10 day post-treatment, and the lung sections were stained with hematoxylin and eosin (***A:*** The control group, 200 ×, ***B:*** The bleomycin (BLM) group, 400 ×, ***C:*** bleomycin + RSV group, 400 ×).

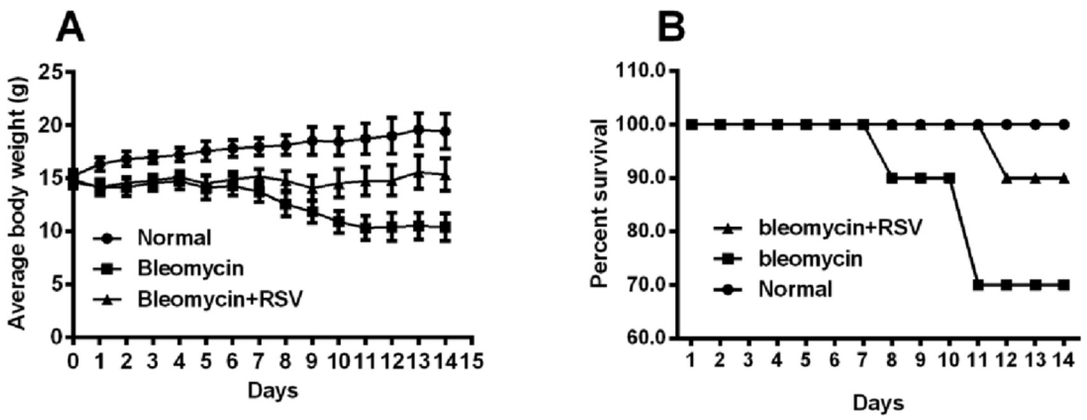

Figure 2. Body weight change and survival of mice treated with bleomycin (and) resveratrol. Mice (average body weight of 15.103 ± 0.571 g, N = 10 in each group) were intratracheally injected with 50 μl per mouse physiological saline (normal control), with 100 μg bleomycin per mouse (dissolved in 50 μl physiological saline) (bleomycin group) or with both 100 μg bleomycin and 50 μg resveratrol per mouse (dissolved in 50 μl physiological saline) (bleomycin + RSV group) at 0 day. Then the mice body weight ***(A)*** and the survival ***(B)*** were monitored daily. The mean titer and standard deviation (error bar) were calculated from the 10 mice in each group. The data were analyzed with two-way ANOVA, and statistical significance was considered when $p < 0.05$ or less.

that there was a significant downregulation of Sirt 1 mRNA level in the BLM-treated mice lung (p <0.01 for column 2 *vs*. 1 and $p < 0.001$ for column 5 *vs*. 4 in Figure 3A). However, such Sirt 1 downregulation in mRNA level was markedly inhibited by the resveratrol treatment (p <0.05 for column 3 *vs*. 2 and $p < 0.001$ for column 6 *vs*. 5 in Figure 3A). In addition, we also examined the Sirt 2 mRNA level in each group of mice. As shown in Figure 3B, there was no significant difference in Sirt 2 mRNA among the three groups at 5 day post-treatment; however, the Sirt 2 mRNA was also markedly lower in the BLM& resveratrol group than in the BLM group (p < 0.05 for column 5 *vs*. 4 in Figure 3B).

Western blotting assay was also performed to examine the protein level of Sirt 1 in each group. Figure 3C demonstrated that the downregulation of Sirt 1 was also confirmed in the BLM group ($p < 0.01$ for column 2 *vs*. 1 or 5 *vs*. 4 at 5 or 10 day post-treatment). And such downregulation was also markedly ameliorated by the resveratrol treatment ($p < 0.05$ for column 3 *vs*. 2 or 6 *vs*. 5 at 5 or 10 day post-treatment). However, no difference was observed among the three groups in the protein level of Sirt 2 (Figure 3D). In addition, we determined the Sirt 1 activity in each group of mice. And results demonstrated that the Sirt 1 activity was significantly downregulated in BLM group, whereas also markedly ameliorated in the BLM & resveratrol group ($p < 0.01$ for column 2 *vs*. 1 or 5 *vs*. 4, or $p < 0.05$ for column 3 *vs*. 2 or 6 *vs*. 5 at 5 or 10 day post-treatment).

Resveratrol inhibits the BLM-induced epithelial-to-mesenchymal transition (EMT) in mice lung

To investigate the regulation by BLM or (and) Resveratrol on the fibrosis or EMT in mice lung, we analyzed the EMT-associated markers, such as E-cadherin, collagen I and α-SMA in the lung of mice, which were treated with BLM or (and) resveratrol. Western blotting (Figure 4A) demonstrated that the E-cadherin was markedly downregulated in mice lung at 5 or 10 day post the BLM treatment ($p <$ 0.001 for column 2 *vs*. 1 and $p < 0.01$ for column 5 *vs*. 4, Figure 4B); and such downregulation was significantly inhibited by resveratrol ($p < 0.01$ for column 3 *vs*. 2 or $p < 0.05$ for Column 6 *vs*. 5 at 5 or 10 day post-treatment, Figure 4B). However, the levels of collagen I and α-SMA were significantly upregulated by the BLM treatment ($p < 0.05$ for column 2 *vs*. 1 and for column 5 *vs*. 4, Figure 4C and 4D). Moreover, such upregulation was also inhibited by resveratrol ($p < 0.05$ or $p < 0.01$ for column 3 *vs*. 2 or $p < 0.05$ for column 6 *vs*. 5 at 5 or 10 day post-treatment, Figure 4C and 4D). Taken together, BLM promotes the EMT-associated markers in mice lung, whereas resveratrol inhibits such promotion.

Discussion

Fibroblasts are main sources for the abnormally elevated synthesis and deposition of collagen and matrix in pulmonary fibrosis[19]. And the origin of lung fibroblasts during pulmonary fibrosis include the proliferation of resident lung interstitial fibrob-

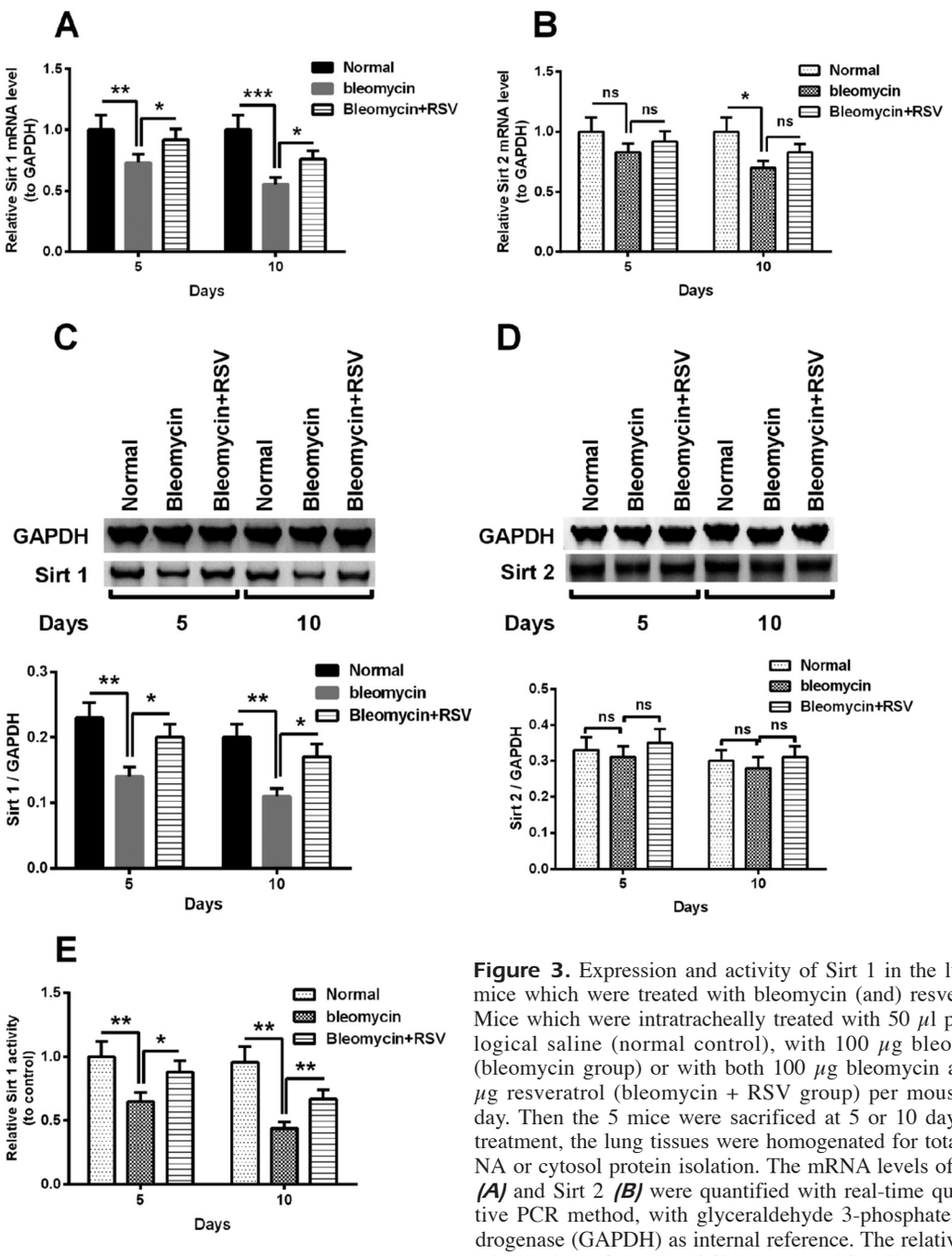

Figure 3. Expression and activity of Sirt 1 in the lung of mice which were treated with bleomycin (and) resveratrol. Mice which were intratracheally treated with 50 μl physiological saline (normal control), with 100 μg bleomycin (bleomycin group) or with both 100 μg bleomycin and 50 μg resveratrol (bleomycin + RSV group) per mouse at 0 day. Then the 5 mice were sacrificed at 5 or 10 day post-treatment, the lung tissues were homogenated for total mRNA or cytosol protein isolation. The mRNA levels of Sirt 1 ***(A)*** and Sirt 2 ***(B)*** were quantified with real-time quantitative PCR method, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal reference. The relative protein levels to GAPDH of Sirt 1 ***(C)*** and Sirt 2 ***(D)*** and the Sirt 1 activity ***(E)*** were quantified with western blotting assay or with SIRT1 Activity Assay Kit. The mean value and standard deviation (error bar) were calculated from the results of five mice. The data were analyzed with two-way ANOVA and unpaired *t*-test. The *p*-values were indicated as (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ or (ns) no significance.

lasts, differentiation of progenitor cells from the bone marrow, and transition of epithelial cells to a fibroblast phenotype, a process termed epithelial–mesenchymal transition (EMT)[20]. In particular, EMT is increasingly recognized as one key step to develop the idiopathic pulmonary fibrosis, which is caused by drugs, such as BLM, with convincing evidence *in vivo* in murine models of pulmonary fibrosis[21]. However, the knowledge about signaling pathways underlining the EMT and fibrosis is unclear. And current therapeutic strategies are primarily aimed at controlling the inflammatory processes, and are often limited to the oral administration of glucocorticosteroids, since the 1950s[22].

In the present study, we evaluated the therapeutic role of resveratrol in the BLM-induced fibrosis in a mouse model. We confirmed the promotion to pulmonary fibrosis by BLM in mice with the pathological evidence in lung, the mice body weight loss and death caused by the agent. However, the pathological change of pulmonary fibrosis was ameliorated by resveratrol, and the body weight loss and death of BLM-treated mice were also markedly ameliorated by resveratrol. Moreover, we confirmed the inhibition by resveratrol in the BLM-induced EMT in mice lung. The typical EMT molecular events, such as E-cadherin downregulation and the upregulation of collagen I and α-SMA, were confirmed by western blot analysis. However, such typical EMT molecular events were also inhibited by resveratrol.

Resveratrol is primarily found in such natural food as grapes, red wine, and peanuts[23]. Resveratrol has been indicated to regulate a variety of cellular responses like cell cycle arrest, differentiation, and apoptosis in various tumor cell lines[24], acting as a potent activator of sirtuin activity[25]. In our study, we examined the expression and activity of Sirt 1 in the mice treated with BLM and resveratrol. Our results demonstrated the marked inhibition by BLM in both mRNA and protein levels in mice lung. And the Sirt 1 activity was also inhibited by the BLM treatment. However, such inhibition was significantly ameliorated by the treatment with resveratrol. The Sirt 1 in mRNA and protein levels and the Sirt 1 activity were markedly ameliorated by the resveratrol treatment in mice lung.

Conclusions

Our study confirmed the inhibitory role of resveratrol in the bleomycin-induced pulmonary fibrosis in a mouse model. Resveratrol ameliorat-

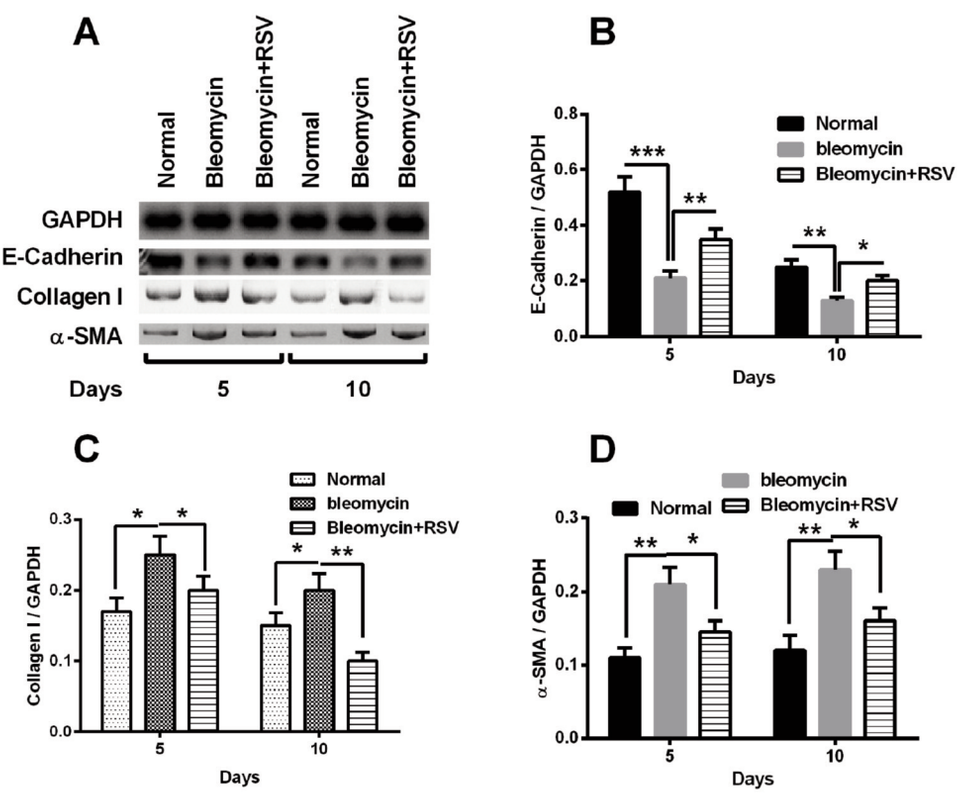

Figure 4. Regulation by the treatment with Bleomycin (and) Resveratrol in mice lung of epithelial-to-mesenchymal transition (EMT)-associated markers. Five mice which were intratracheally treated with 50 μl physiological saline (Normal control), with 100 μg Bleomycin (Bleomycin group) or with both 100 μg Bleomycin and 50 μg Resveratrol (Bleomycin + RSV group) were sacrificed at 5 or 10 day post-treatment, the lung tissues were homogenated for cytosol protein isolation. The protein levels of E-Cadherin, Collagen I, α-SMA and GAPDH were analyzed with western blotting assay ***(A)***. The relative level of E-Cadherin ***(B)***, Collagen I ***(C)***, α-SMA ***(D)*** to GAPDH was indicated respectively. The mean value and standard deviation (error bar) were calculated from the results of five mice. The data were analyzed with two-way ANOVA and unpaired t-test. The p-values were indicated as (*) p <0.05, (**)p <0.01 or (***)p <0.001.

ed the BLM-induced pathological change of fibrosis, mice body weight loss and death. And such amelioration might be associated with the activation of Sirt 1 and with the expression of EMT-associated markers in mice lung. The present study implied that resveratrol might be a promising agent for effective control the pulmonary fibrosis.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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