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Hydrogen sulfide attenuates lung ischemia–reperfusion injury through SIRT3-dependent regulation of mitochondrial function in type 2 diabetic rats

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ABSTRACT

Background: Lung ischemia–reperfusion injury is a complex pathophysiologic process associated with high morbidity and mortality. We have demonstrated elsewhere that diabetes mellitus aggravated ischemia-induced lung injury. Oxidative stress and mitochondrial dysfunction are drivers of diabetic lung ischemia-reperfusion injury; however, the pathways that mediate these events are unexplored. In this study using a high-fat diet–fed model of streptozotocin-induced type 2 diabetes in rats, we determined the effect of hydrogen sulfide on lung ischemia-reperfusion injury with a focus on Sirtuin3 signaling.

Methods: Rats with type 2 diabetes were exposed to GYY4137, a slow release donor of hydrogen sulfide with or without administration of the Sirtuin3 short hairpin ribonucleic acid plasmid, and then subjected to a surgical model of ischemia–reperfusion injury of the lung ($n = 8$). Lung function, oxidative stress, inflammation, cell apoptosis, and mitochondrial function were measured.

Results: Compared with nondiabetic rats, animals with type 2 diabetes at baseline exhibited significantly decreased Sirtuin3 signaling in lung tissue and increased oxidative stress, apoptosis, inflammation, and mitochondrial dysfunction ($P < .05$ each). In addition, further impairment in Sirtuin3 signaling was found in diabetic rats subjected to this model of lung ischemia–reperfusion. Simultaneously, the indexes showed further aggravation. Treatment with hydrogen sulfide restored Sirtuin3 expression and decreased lung ischemia–reperfusion injury in animals with type 2 diabetes mellitus by improving lung functional recovery, decreasing oxidative damage, suppressing inflammation, ameliorating cell apoptosis, and preserving mitochondrial function ($P < .05$). Conversely, these protective effects were largely reversed in Sirtuin3 knockdown rats.

Conclusion: Impaired lung Sirtuin3 signaling associated with type 2 diabetic conditions was further attenuated by an ischemia-reperfusion insult. Hydrogen sulfide ameliorated reperfusion-induced oxidative stress and mitochondrial dysfunction via activation of Sirtuin3 signaling, thereby decreasing lung ischemia-reperfusion damage in rats with a model of type II diabetes.

Introduction

Ischemia-reperfusion–induced lung injury is commonly encountered in hospital and in outpatient settings and is associated with a high rate of morbidity and mortality. Many clinical events, for instance, pulmonary embolism, lung transplantation, and cardiopulmonary bypass, can contribute to lung ischemia-reperfusion (I/R) injury.[1] Diabetes mellitus (DM) is a rapidly growing health concern associated with many severe complications. Increasing

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evidence has demonstrated that the lung is a target of diabetic injury, but the lung is one of the least-studied organs regarding diabetic complications.[2,3] Recently, an unexpectedly high rate of DM and prediabetes was diagnosed in patients awaiting lung transplantation. This is important, because DM is an independent risk factor for 5-year mortality after lung transplantation.[4] We demonstrated elsewhere that DM aggravated lung I/R injury and oxidative stress played a key role in this process.[5] Of note, because mitochondria are a major source of and target organ for reactive oxygen species (ROS), mitochondrial dysfunction is recognized as a key factor contributing to I/R injuries under diabetic conditions.[6,7] Therefore, strategies to target the sources of ROS and to preserve mitochondrial function are attractive approaches to abate I/R injury in the diabetic state.[8,9]

Hydrogen sulfide (H_2S) has become recognized as a crucial signaling molecule contributing to many physiologic and pathologic processes.[10] Circulating levels of H_2S are decreased in patients with type 2 DM and in diabetic animal models and appear to contribute to the pathophysiology of diabetes.[11,12] Recently, therapeutic strategies to increase levels of H_2S appear to be promising approaches to ameliorate myocardial I/R-induced tissue injury in the diabetic state, but the underlying mechanisms remain poorly defined.[12,13] Our research, which has been published elsewhere, confirmed that H_2S was able to attenuate lung I/R injury by decreasing oxidative damage and ameliorating mitochondrial injury.[14] The effect and mechanism of H_2S treatment on lung I/R injury under type 2 diabetic conditions, however, are still not completely clear.

Sirtuin3 (SIRT3) is a sirtuin family member that is localized mainly in the mitochondria.[15] By deacetylating proteins, such as manganese superoxide dismutase (SOD2), cyclophilin D (CypD), and Ku70, SIRT3 enhances mitochondrial antioxidant defenses and protects mitochondrial function.[16–18] Because mitochondria are sensitive targets for I/R injury, the role of SIRT3 in protection against I//R injury has attracted increasing interest.[19,20] Of note, impaired SIRT3 signaling was found in the mitochondria of diabetic lung tissue, indicating an abnormality in mitochondrial function, which contributes to lung injury in diabetes.[2] Recently, SIRT3 has emerged as an effective protector against I/R-induced tissue injury in the diabetic state.[7,21] Moreover, several studies have suggested that H_2S could upregulate SIRT3 activity and expression to exert either physiologic or pathologic effects.[22–24] Nevertheless, alteration of lung SIRT3 signaling in animals with type 2 diabetes subjected to lung I/R injury and its association with mitochondrial function or H_2S is still unknown.

We hypothesized that H_2S might play a protective role in diabetic lung I//R injury and that SIRT3 signaling plays a regulatory role in this process. On the basis of our observations, we used GYY4137, a slow-releasing H_2S donor to (1) investigate the effect of H_2S treatment on lung I/R injury in a rat model of type 2 diabetes and (2) determine the potential roles of SIRT3 signaling in H_2S treatment in this same rat model of type 2 DM.

Materials and Methods

Animals

Pathogen-free, male Sprague Dawley rats, weighing 200 to 220 g, were purchased from Harbin Medical University (Harbin, Heilongjiang, China). All animal experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised, 1996). All animal experiments were approved by the Institutional Animal Care and Use Committee at Harbin Medical University.

Type 2 diabetic rat model

We developed a group of high-fat diet–fed streptozotocin-induced type 2 diabetic male rats, which is a well-recognized diabetic model.[25,26] Therefore, we only used male rats for our study. The rats were fed high-fat food containing (20% sucrose, 5% sesame oil, 15% lard, 2.5% cholesterol, and 57.5% normal chow) for 6 weeks followed by intraperitoneal injection of streptozotocin (35 mg/kg) (Sigma, St. Louis, MO, USA). Thereafter the, rats were fed continuously with high-fat food. The rats with fasting plasma glucose concentrations greater than 11.1 mmol/L at least 72 hours after the streptozotocin injection were classified as diabetic. Rats fed the standard laboratory chow were studied as the nondiabetic controls.

Rat lung I/R model

The lung I/R model was established as described elsewhere.[5] The rats were anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg; Shougang Biotechnological Company, Harbin, China), intubated through a tracheostomy, and ventilated with 40% oxygen (with balanced nitrogen) at a tidal volume of 10 ml/kg with 2 cmH_2O positive end-expiratory pressure (PEEP). The breathing rate was adjusted to maintain arterial carbon dioxide tension ($PaCO_2$) between 35 mmHg to 45 mmHg. The right femoral arteries were catheterized for pressure monitoring (Datex, Helsinki, Finland) and arterial blood sampling analysis (Bayer, Medfield, MA, USA), and the right femoral vein was catheterized for drug administration. After a left thoracotomy, the hilum of the left lung was clamped with a noncrushing microclip at the end of expiration 5 minutes after the administration of heparin (50 IU/animal). Subsequently, the tidal volume was adjusted to 6 mL/kg. The hilum of the left lung was clamped for 90 minutes and released, reperfusion occured for 4 hours, and the tidal volume was increased to 10 mL/kg. All rats were positioned on a heating pad to maintain body temperature, muscle relaxation was maintained with intravenous pipecuronium bromide (0.4 mg $kg^{-1}h^{-1}$), and sodium pentobarbital was used to maintain anesthesia. Rats in the sham groups underwent the same procedure except for the clamping of the hilum of the left lung.

Experimental groups

The rats were divided randomly into the following six groups: sham group (Con+Sham, $n = 8$), lung I/R group (Con+I/R, $n = 8$), DM+ sham group (DM+Sham, $n = 8$), DM+ lung I/R group (DM+I/R, $n = 8$), DM+ I/R+GYY4137-treated group (DM+I/R+H, $n = 8$), and DM+ I/R+GYY4137 and SIRT3 shRNA treated group (DM+I/R+H+S, $n = 8$). GYY4137 (133 μmol/kg, dissolved in 1.0 ml of sterile 154 mM NaCl) was injected intraperitoneally 1 hour before the start of the operative model of lung I/R injury. SIRT3 short hairpin ribonucleic acid (shRNA) plasmid at a dose of 2 mg/kg was injected intratracheally into the rat lung through a 16-gauge oral catheter (to selectively downregulate lung SIRT3 expression). The doses of GYY4137 and SIRT3 shRNA plasmid were selected on the basis of our earlier reports.[27,28]

Preparing the SIRT3 shRNA expression vector

Rat SIRT3 was treated as the target gene and shRNA oligonucleotides using pcDNATM6.2-GW/EmGFP-miR as an expression vector were designed and synthesized by Gene Pharma (Shanghai, China). The target-specific sequence was 5'-AATTCGGGTCCTTTGTATCAGC TACGGTTTTGGCCACTGACTGACCGTAGCTGATACAAAGGACCCA-3' (sense) and 5'-GCCCAGGAAACATAGTCGATGCCAAAAC CGGTGACTGACTGG CATCGACTATGTTTCCTGGGTGGCC-3' (antisense)

The scrambled target sequence was 5'-AATTCGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTTCA-3' (sense) and 5'-CCGGTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACGCGCAGTACATTTCG-3' (antisense).

In vitro and in vivo silencing of SIRT3

Rat glioma cells (C6) were purchased from the Institute of Cellular Biology of Chinese Academy of Sciences (Shanghai, China). C6 cells were transfected with SIRT3 shRNA plasmids, using Lipofectamine 2000 (Invitrogen, Rockford, IL, USA) according to standard protocols. Scrambled shRNA plasmids were used as a negative control (NC). Cells were transfected with 1.6 μg of SIRT3 shRNA plasmids or negative control shRNA plasmids and incubated for 48 hours. SIRT3 interference expression levels were assayed by RT-PCR 48 hours after transfection. SIRT3 shRNA plasmid silencing of lung SIRT3 expression in rats was performed as described elsewhere.[28] Rats were intubated with a 16-gauge oral catheter, and 2 mg/kg SIRT3 shRNA plasmid or scrambled shRNA plasmid was injected into the rat's lung through the catheter. After injection, rats were ventilated with 40% oxygen and at a tidal volume of 10 mL/kg with 2 cmH_2OPEEP. Rats were extubated after recovery from the anesthesia.

Isolation of lung mitochondria

Mitochondria from the lung were isolated by using a mitochondria isolation kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions.

Real-time quantitative PCR

Total RNA was extracted and reverse transcription was performed by using a kit (total RNA extraction reagent; Takara, Japan) following the manufacturer's instructions. Primer sequences from Invitrogen were:

SIRT3-F: GGAAAGTCGCACAGGAGATCATC,
R: AGGTCCCTGGTCAGCCTTAACA.
GADPH-F: ATGTTCCAGTATGACTCTA, R: CACCCCATTTGATGTTAG.

The following thermal cycling protocol was used: 95°C for 3 minutes, followed by 40 cycles of amplification at 95°C for 30 seconds and 62°C for 40 seconds.

Western blot analysis

Western blot analysis was carried out as described elsewhere.[5]

Immunohistochemistry analysis

Anti-SIRT3 and anti-SOD2 antibodies were used as the primary antibodies for immunohistochemical staining according to the manufacturer's instructions (Cell Signaling Technology, Boston, MA, USA). A 3,3'-diaminobenzidine (DAB) staining (Zhongshan Golden Bridge Biotechnology, Beijing, China) was used to detect the positive area.

Histologic analysis

The lung tissues were fixed in paraformaldehyde, embedded in paraffin, sectioned (5-μm thickness), and stained with hematoxylin and eosin. The degree of the lung injury was assessed with a histologic study score using the following criteria: (1) neutrophil infiltration, (2) airway epithelial cell damage, (3) interstitial edema, (4) hyaline membrane formation, and (5) hemorrhage. Each criterion was scored on a semiquantitative scale of 0 to 4 as follows: normal = 0, minimal change = 1, mild change = 2, moderate change = 3, and severe change = 4.

Measurement of the static compliance of the lungs and wet-to-dry lung weight ratio

At the time of death, the animals were connected to an apparatus to measure the static pressure-volume (P-V) curves of the lungs after median sternotomy as described elsewhere.[14] In brief, the lung volumes were measured by increases from 0 to 30 cm H_2O and decreases to 0 cm H_2O with 1 minute of stabilization in a stepwise intervals of 5 cm H_2O. The lung volumes were corrected for gas compression in the apparatus. The upper section of the lung was desiccated at 80°C for 72 hour for calculation of the wet weight-to-dry weight ratio (W/D).

Survival analysis

After a 4-hour reperfusion, rats were extubated after recovery from anesthesia, and the survival rate was evaluated daily for the 168-hour (1 week) period. The surviving rats were killed by a lethal dosage of sodium pentobarbital after the 168-hour observation point. The definition of death was cessation of cardiac mechanical activity.

Enzyme-linked immunosorbent assay

Serum concentrations of tumor necrosis factor-α and interleukin-6 were detected by enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Determination of myeloperoxidase, malonaldehyde, SOD, and total antioxidative capability

Myeloperoxidase (MPO) activity, malonaldehyde (MDA) activity, superoxide dismutase (SOD) activity, and total antioxidative capability (T-AOC) levels in lung tissues were measured by commercial kits (Jiancheng Bio-Technology, Nanjing, China) as described elsewhere.[14]

Apoptosis assay

Apoptosis of lung parenchymal cells was measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using an In Situ Cell Death Detection kit (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's instructions. Cells with red nuclear staining were considered positive as were all of the cells with 4′,6-diamidino-2-phenylindole staining. The apoptosis index was calculated as the ratio of the number of apoptotic nuclei to the total number of nuclei counted.

Detection of mitochondrial morphology and membrane potential

The degree of mitochondrial injury was assessed by the Flameng score as follows[29]: 0, structures are normal and particles are intact; 1, structures are normal and particles are lost; 2, mitochondria are swollen but matrices are clear; 3, cristae are broken and matrices are concentrated; 4, cristae are extensively destroyed and the membranes are ruptured. Mitochondrial membrane potential ($\Delta\psi m$) was determined by a JC-1 staining using a kit from Sigma-Aldrich (St. Louis, MO). In healthy cells, JC-1 concentrates in the matrix, where it forms red fluorescent agglomerated JC-1 (590 nm).

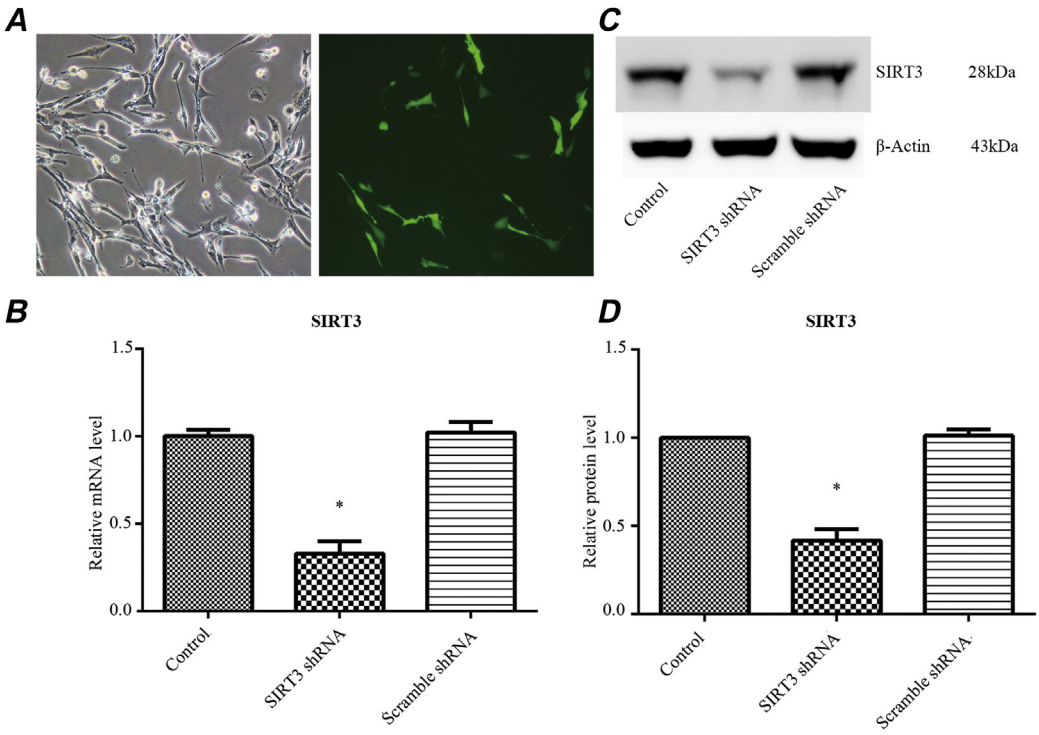

Fig 1. The SIRT3 gene was silenced in vitro and in vivo by SIRT3 short hairpin RNA (shRNA). (*A*) The bright and dark field images of C6 cells after SIRT3 shRNA transfection by fluorescence microscopy (magnification: 100×). (*B*) The interference efficiency of SIRT3 in SIRT3 shRNA-transfected cells using real-time PCR. (*C*) The downregulation of levels of SIRT3 were evaluated by Western blots in vivo. (*D*) SIRT3 expression. SIRT3, Sirtuin3. (*$P < .05$ versus others; $n = 4$ in each group.)

In damaged cells, the dissipated potential of the mitochondrial membrane prevents the accumulation of the JC-1 in the mitochondria, and thus JC-1 is dispersed in the cytoplasm, leading to a shift from aggregated JC-1 to green fluorescent monomeric JC-1 (530 nm). The fluorescence was detected by a fluorimeter (Infinite M200 Pro, Tecan, Switzerland). Mitochondrial membrane potential was expressed as the ratio of aggregated JC-1 to monomeric JC-1.

Statistical analysis

The data are expressed as the means ± standard deviation (SD). Statistical testing was performed using the Prism software package (v 5.0, GraphPad Software, La Jolla, CA, USA). Differences were compared by one-way ANOVA followed by Tukey test for multiple comparisons. For survival analysis, the Kaplan-Meier method was used followed by a log-rank (Mantel-Cox) test.

Results

Downregulation of SIRT3 expression in vitro and in vivo

To explore the role of H_2S on diabetic lung I/R injury and to investigate the underlying mechanisms, we used the SIRT3 shRNA plasmid in vitro and in vivo. We measured SIRT3 expression by RT-PCR in vitro and by Western blot in vivo to evaluate whether SIRT3 could be silenced in both transcriptional and posttranscriptional fields by the designed SIRT3 shRNA. In the in vitro transfection, the expression of SIRT3 mRNA in C6 cells was markedly decreased after treatment with SIRT3 shRNA compared with the expression of SIRT3mRNA after treatment with scrambled shRNA ($P < .05$; Fig 1, *A*). Similar decreases in the expression of SIRT3mRNA were observed in the SIRT3 shRNA group ($P < .05$, compared with the empty vector group; Fig 1, *B*). In the in vivo transfection, the expression of lung SIRT3 protein was dramatically decreased in the SIRT3 shRNA group compared with that in the scrambled shRNA group ($P < .05$; Fig 1, *C*). Similar decreases in the expression of SIRT3 protein were observed in the SIRT3 shRNA group ($P < .05$, compared with the empty vector group; Fig 1, *D*).

SIRT3 signaling pathway impaired under diabetic conditions and further attenuated when subjected to lung I/R

We measured the SIRT3 signaling in both diabetic and nondiabetic animals by Western blot and immunohistochemistry staining. Figure 2 shows that the expression level of SIRT3 was dramatically decreased in the DM+Sham group compared with the Con+Sham group ($P < .05$). The expression level of SIRT3 was also attenuated in the Con+I/R group compared with that in the Con+Sham group ($P < .05$). The expression level of SIRT3 was further attenuated in the DM+I/R group compared with that in the Con+I/R group ($P < .05$). Compared with that in the DM+ Sham group, the expression level of SIRT3 in the DM+I/R group also showed a decrease ($P < .05$). The H_2S treatment restored the expression level of SIRT3 considerably ($P < .05$, compared with the DM+I/R group), and these effects were partially abolished by treatment with the SIRT3 shRNA plasmid ($P < .05$, compared with the DM+I/R+H group). Similar changes were observed in the Western blot and immunohistochemistry staining for SOD2 (Fig 2, *A*, *C*, and *F*); however, acetylation of SOD2 showed contrasting tendencies. As shown in Fig 2, the acetylation of SOD2 was increased dramatically in the DM+Sham group compared with the Con+Sham group ($P < .05$), and acetylation of SOD2 was also increased in the Con+I/R group compared with that in the Con+Sham group ($P < .05$). The acetylation of SOD2 was further

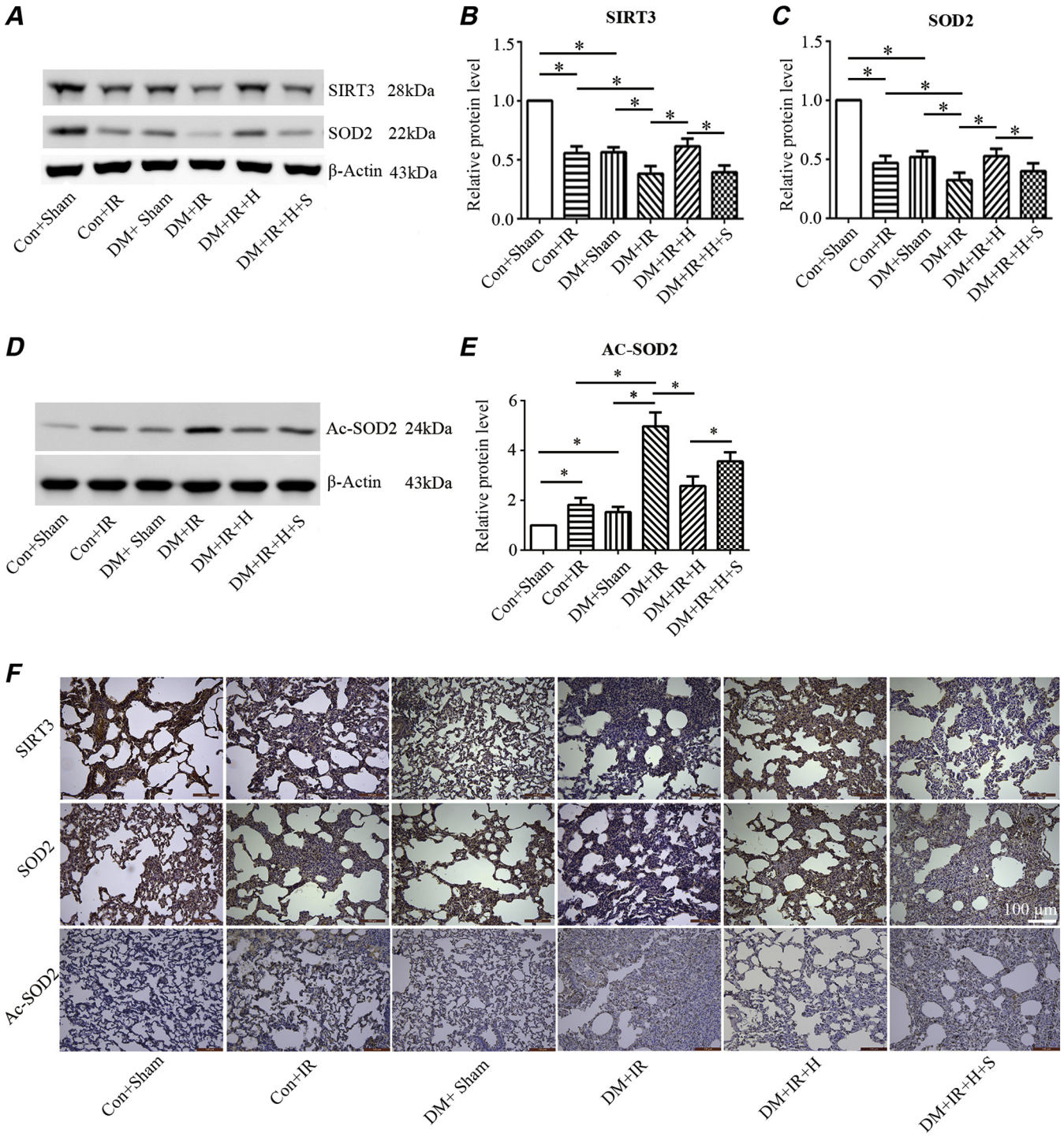

Fig 2. Type 2 DM impaired lung SIRT3 signaling was further attenuated when subjected to I/R insult. H_2S restored SIRT3 expression. (*A*) Representative blots. (*B*) SIRT3 expression. (*C*) SOD2 expression. (*D*) Representative blots. (*E*) Ac-SOD2 expression. (*F*) Representative lung immunohistochemical images of SIRT3, SOD2, and Ac-SOD2 (magnification: 200×). ($^*P < .05$ versus others; $n = 8$ in each group). SIRT3, Sirtuin3; SOD2, manganese superoxide dismutase; Ac-SOD2, acetylated manganese superoxide dismutase; I/R, ischemia-reperfusion; DM, diabetes mellitus.

increased in the DM+I/R group compared with that in the Con+I/R group ($P < .05$). Similar tendencies were observed in the DM+I/R group ($P < .05$, compared with the DM+Sham group). The H_2S treatment, however, markedly decreased the acetylation of SOD2 expression ($P < .05$, the DM+I/R+H group compared with the DM+I/R group). These effects were partially abolished by SIRT3 shRNA plasmid treatment ($P < .05$, compared with the DM+I/R+H group) (Fig 2, *D–F*).

SIRT3 signaling pathway participates in H_2S-mediated pulmonary protection of diabetic lung I/R injury

Hypoxemia is a hallmark of lung injury. As presented in Fig 3 (*A*) and Table I, the arterial blood gas analysis showed that the oxygenation index (PaO_2/FiO_2) was decreased in the DM+Sham group compared with that in the Con+Sham group ($P < .05$). Similar decreases were observed in the Con+I/R group ($P < .05$,

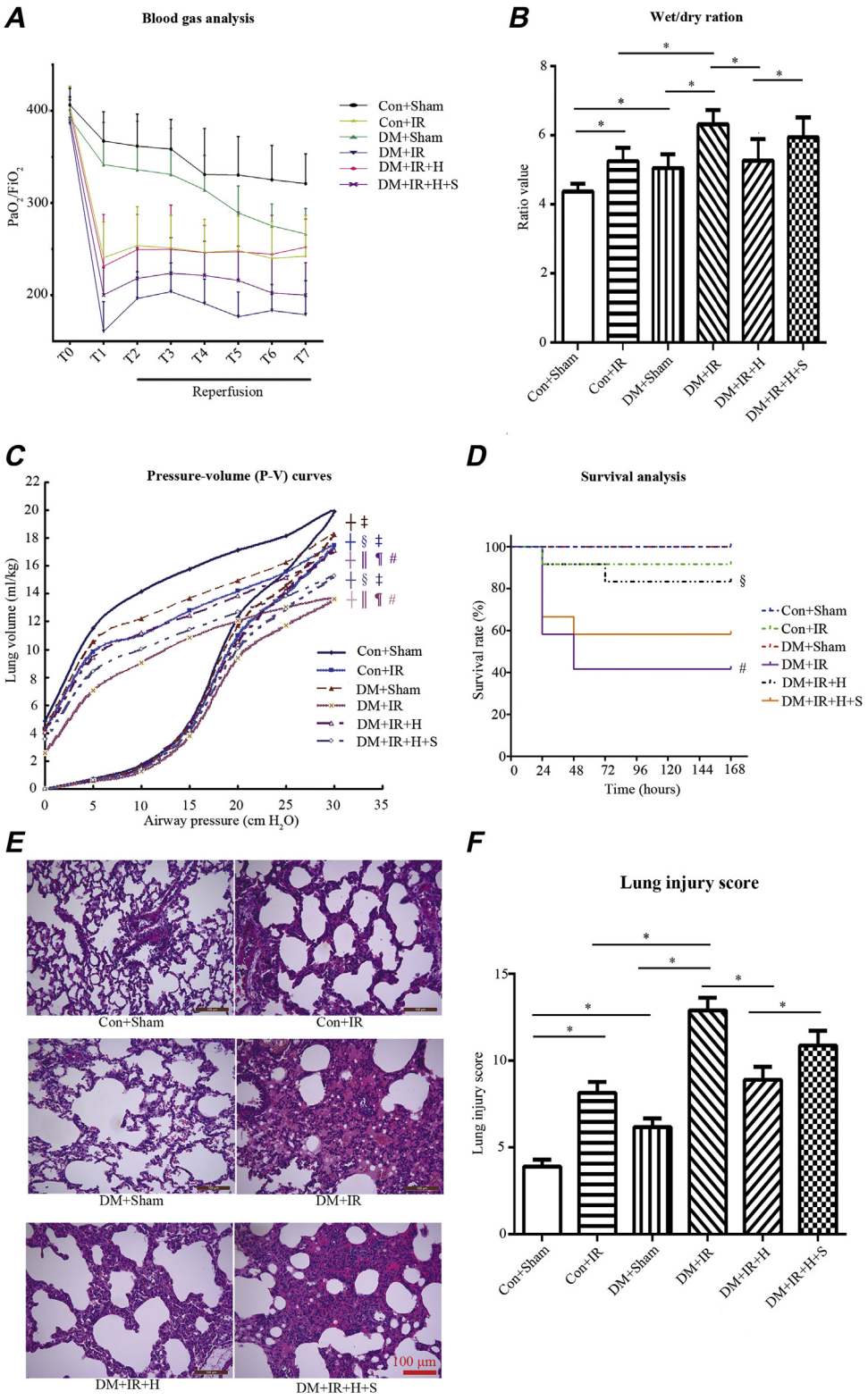

Fig 3. H_2S conferred a pulmonary protective effect against lung I/R injury in rats with type 2 diabetes mediated by SIRT3 signaling. (*A*) Arterial blood gas analysis. T0-T7 represented the following time points: baseline; the end of ischemia; and 30, 60, 90, 120, 180, and 240 minutes after reperfusion. (*P* values are presented in the Table I.) (*B*) Wet-to-dry weight ratio. (*C*) Static compliance of the lung pressure-volume (P-V) curves. Data are presented as the mean values, and the bars are omitted for clarity. (*D*) Survival analysis. Rats were observed for 168 hours (1 week) and survival time was calculated (n = 12). (*E*) Histologic analysis of lung tissues (magnification: 200×). (*F*) Lung injury score. PaO_2/FiO_2: partial pressure of arterial oxygen (PaO_2)/fraction of inspired oxygen (FiO_2). T0–T7 represented the following time points: baseline; the end of ischemia and 30, 60, 90, 120, 180, and 240 minutes after reperfusion. ($^{*}P < .05$ versus others, $^{\dagger}P < .05$ versus Con+sham group, $^{\ddagger}P < .05$ versus DM+Sham group, $^{\S}P < .05$ versus DM+I/R group, $^{\|}P < .05$ versus DM+I/R+H group, $^{\P}P < .05$ versus DM+I/R+H+S group, $^{\#}P < .05$ versus Con+I/R group; $n = 8$ in each group except for survival analysis).

Table I
Arterial blood gas analysis

	Group	T0	T1	T2	T3	T4	T5	T6	T7
PaO_2/FiO_2 (mmHg)	Con+Sham	406 ± 18	367 ± 32	362 ± 35	359 ± 32	331 ± 50	330 ± 42	325 ± 37	321 ± 32
	Con+I/R	405 ± 21	241 ± 39[*,†,‡]	254 ± 42[*,†]	251 ± 35[*,†]	247 ± 36[*,†,‡]	249 ± 37[*,‡]	240 ± 40[*,‡]	242 ± 44[*,‡]
	DM+Sham	392 ± 22	342 ± 46[‡,§,‖,¶]	336 ± 52[‡,§,‖,¶]	331 ± 50[‡,§,‖,¶]	314 ± 37[‡,§,‖,¶]	289 ± 29[‡,¶]	275 ± 24[‡,¶]	266 ± 28[*,‡,¶]
	DM+I/R	386 ± 26	161 ± 32[*,†,§,‖]	196 ± 29[*,†]	204 ± 31[*,†]	191 ± 26[*,†,§,‖]	177 ± 27[*,†,§,‖]	183 ± 28[*,†,§,‖]	179 ± 37[*,†,§,‖]
	DM+I/R+H	401 ± 25	231 ± 56[*,†,‡]	250 ± 38[*,†]	250 ± 48[*,†]	246 ± 29[*,†,‡]	247 ± 43[*,‡]	245 ± 42[†,‡]	252 ± 30[*,‡,¶]
	DM+I/R+H+S	391 ± 24	200 ± 29[*,†]	218 ± 33[*,†]	224 ± 38[*,†]	221 ± 37[*,†]	216 ± 37[*,†]	202 ± 42[†,§]	200 ± 35[*,†,‖]

Note: T0–T7 represented the following time points: baseline; the end of ischemia; and 30, 60, 90, 120, 180, and 240 minutes after reperfusion. PaO_2/FiO_2: partial pressure of arterial oxygen (PaO_2)/fraction of inspired oxygen (FiO_2). Results are expressed as mean ± SD.

[*] $P < .05$ versus Con+sham group
[†] $P < .05$ versus DM+Sham group
[‡] $P < .05$ versus DM+I/R group
[§] $P < .05$ versus Con+I/R group
[‖] $P < .05$ versus DM+I/R+H group
[¶] $P < .05$ versus DM+I/R+H+S group ($n = 8$ in each group)

compared with the Con+Sham group). Compared with that in the Con+I/R group, the PaO_2/FiO_2 in the DM+I/R group showed a further decrease ($P < .05$). Compared with that in the DM+Sham group, the PaO_2/FiO_2 in the DM+I/R group also showed a decrease ($P < .05$). The H_2S treatment (DM+I/R+H group) improved the level of PaO_2/FiO_2 compared with that in the DM+I/R group ($P < .05$); however, these effects were partially abolished by treatment with the SIRT3 shRNA plasmid ($P < .05$, compared with the DM+I/R+H group). The results of blood gas analysis of the left pulmonary venous circulation exhibited the same trend as the arterial blood gas analysis (Table II). As expected, increases in wet weight-to-dry weight ratio, an indicator of lung edema, were correlated with the decreases in PaO2-to-FiO2 ratios (Fig 3, *B*).

We also measured the static compliance of the lung to determine the degree of changes in lung aeration. As shown in Fig 3 (*C*), the volumes in the P-V curve in the Con+Sham group and DM+Sham group showed no difference ($P > .05$). In contrast, the volumes in the P-V curve in the Con+I/R group were less than those in the Con+Sham group ($P < .05$). The volumes in the P-V curve in the DM+I/R group were further decreased compared with those in the Con+I/R group ($P < .05$). Similar decreases in the volumes of the P-V curve were observed in the DM+I/R group ($P < .05$, compared with the DM+Sham group). The volumes in the P-V curve in the DM+I/R+H group were greater than those in the DM+I/R group ($P < .05$), and the DM+I/R+H+S group also showed lesser volumes ($P < .05$, compared with the DM+I/R+H group).

As shown in Fig 3 (*D*), the survival rate was decreased in diabetic rats compared with nondiabetic rats after the model of lung I/R ($P < 0.05$, the DM+I/R group compared with the Con+I/R group). H_2S treatment improved the survival of rats subjected to I/R in the type 2 diabetic rats ($P < .05$, compared with the DM+I/R group). The survival rate in the DM+I/R+H group and DM+I/R+H+S group showed no difference ($P = .17$).

As shown in Figs. 3 (*E* and *F*), the lung morphologies in the Con+Sham group exhibited a relatively normal histology. The Con+I/R group showed interstitial thickening, edema in the alveolar septa and spaces, leukocyte infiltration, and intra-alveolar hemorrhage, ($P < .05$, compared with the Con+Sham group), and more serious histologic changes were present in the DM+I/R group ($P < .05$, compared with the Con+I/R group). More serious histologic changes were also present in the DM+I/R group ($P < .05$, compared with the DM+Sham group). Administration of the H2S donor GYY4137 ameliorated the histologic changes ($P < .05$, compared with the DM+I/R group); whereas SIRT3 shRNA plasmid treatment aggravated the histologic changes ($P < .05$, compared with the DM+I/R+H group).

Table II
The indices of blood gas analysis from pulmonary vein

Group	PvO_2/FiO_2 (mmHg)	$PvCO_2$ (mmHg)
Con+Sham	310 ± 22	40.1 ± 2.7
Con+I/R	251 ± 30[*,†]	40.8 ± 2.8
DM+Sham	262 ± 27[*,†,‡]	39.3 ± 3.8
DM+I/R	191 ± 35[*,§,‖]	38.1 ± 2.4
DM+I/R+H	258 ± 29[*,†,‡]	39.5 ± 3.4
DM+I/R+H+S	212 ± 34[*,‖,¶]	40.4 ± 2.8

Note: After 4 hours of reperfusion, the blood samples were collected from the pulmonary vein used for blood gas analysis. *PvO_2/FiO_2*, pulmonary venous oxygen tension (PvO_2)/fraction of inspired oxygen (FiO_2); *$PvCO_2$*, venous carbon dioxide tension. Results are expressed as mean ± SD.

[*] $P < .05$ versus Con+sham group
[†] $P < .05$ versus DM+I/R group
[‡] $P < .05$ versus DM+I/R+H+S group ($n = 8$ in each group)
[§] $P < .05$ versus Con+I/R group
[‖] $P < .05$ versus DM+Sham group
[¶] $P < .05$ versus DM+I/R+H group ($n = 8$ in each group)

SIRT3 signaling pathway participates in anti-inflammatory and antioxidative effects of H_2S on diabetic lung I/R injury

As shown in Figs 4 (*A* and *B*), serum concentrations of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF- α) in the DM+Sham group were greater than those in the Con+Sham group ($P < .05$). Similar increases in these inflammatory markers were observed in the Con+I/R group ($P < .05$, compared with the Con+Sham group). The levels of TNF-α and IL-6 in the DM+I/R group were further increased compared with those in the Con+I/R group ($P < .05$). Similar increases were observed in the DM+I/R group ($P < .05$, compared with the DM+Sham group), and the levels of these inflammatory markers were less in the DM+I/R+H group than in the DM+I/R group ($P < .05$). Treatment with the SIRT3 shRNA plasmid partially abolished these effects by increasing the levels of TNF-α and IL-6 ($P < .05$, compared with the DM+I/R+H group). The level of MPO, an indicator of neutrophil infiltration, and the level of MDA, a common indicator of oxidative damage, exhibited the same changes as the levels of the inflammatory markers (Figs 4, *C* and *D*). The antioxidative capacity (activities of SOD and T-AOC) showed the opposite changes compared with the previous levels (Figs 4, *E* and *F*).

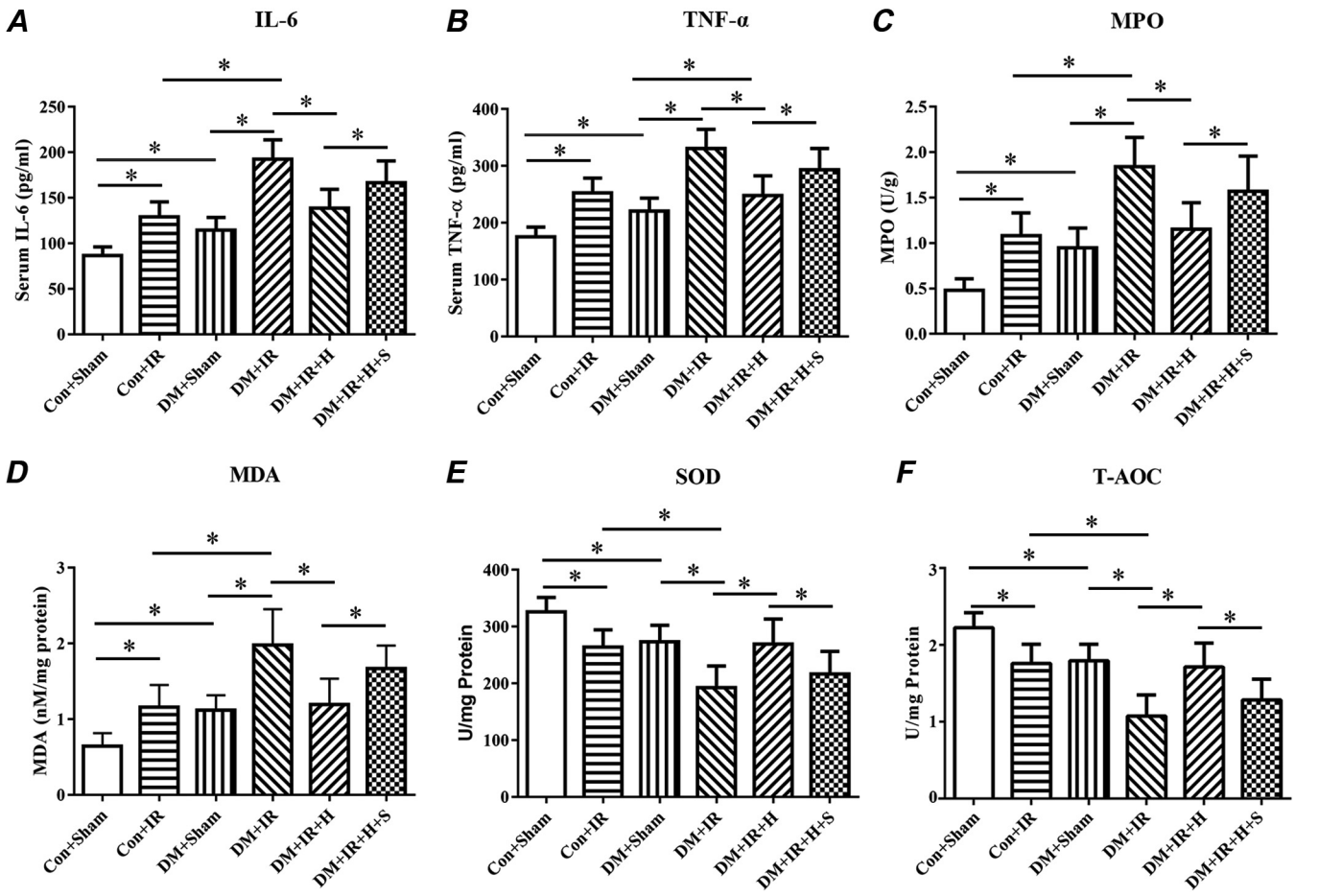

Fig 4. SIRT3 mediated the anti-inflammation and antioxidative stress of H_2S in diabetic lung I/R injury. (*A*) Serum concentrations of interleukin-6 (IL-6) and (*B*) TNF-α and lung concentrations of (*C*) MPO, (*D*) MDA, (*E*) SOD, and (*F*) T-AOC. ($^*P < .05$ versus others; $n = 8$ in each group). IL, Interleukin; TNF-a, tumor necrosis factor-a; MPO, myeloperoxidase; MDA, malonaldehyde; SOD, superoxide dismutase; T-AOC, total antioxidative capability.

SIRT3 signaling pathway participates in H_2S-mediated antiapoptotic effects on diabetic lung I/R injury

As shown in Figs 5 (*A* and *B*), the apoptotic index was increased in the DM+ Sham group ($P < .05$, compared with the Con+Sham group). Similar increases were observed in the Con+I/R group ($P < .05$, compared with the Con+Sham group). Cell apoptosis was also further aggravated in the DM+I/R group ($P < .05$, compared with the Con+I/R group). Compared with the DM+Sham group, cell apoptosis was also increased in the DM+I/R group ($P < .05$). Administration of the H2S donor GYY4137 alleviated cell apoptosis compared with the levels of apoptosis in the DM+I/R group ($P < .05$). The SIRT3 shRNA plasmid treatment partially abolished this effect of H_2S by increasing the apoptotic index ($P < .05$, compared with the DM+I/R+H group). Similar changes were observed in the Western blot for cleaved caspase 3, a key enzyme involved in execution of apoptosis and the proapoptotic factors bax (Fig 5, *C*). Notably, the expression level of mitochondrial bax showed similar changes (Fig 5, *D*). In contrast, the antiapoptosis factor Bcl-2 exhibited the opposite changes (Fig 5, *C*).

SIRT3 signaling pathway participates in protection of mitochondrial function as mediated by H_2S on diabetic lung I/R injury

As shown in Fig 6 (*A*), at the ultrastructural level, the integrity of the mitochondria (without swelling or cristae breakage) was maintained in the Con+Sham group. The mitochondria in the Con+I/R group and DM+sham group were swollen, but the matrices were clear. The mitochondria in the DM+I/R group showed severe swelling with disrupted cristae and large amounts of vacuolation. Administration of the H2S donor GYY4137 resulted in a decrease in swollen mitochondria and disrupted cristae; whereas treatment with the SIRT3 shRNA plasmid partially abolished these effects. Figure 6 (*B*) shows that the mitochondrial Flameng scores of the DM+Sham groups were greater than the scores of the Con+sham group ($P < .05$). Similar increases were observed in the Con+I/R group ($P < .05$, compared with the Con+Sham group). The mitochondrial Flameng score of the DM+I/R group was further increased ($P < .05$, compared with the Con+I/R group). Similar increases were observed in the DM+I/R group ($P < .05$, compared with the DM+Sham group), the GYY4137 treatment decreased the score ($P < .05$, compared with the DM+I/R group), and treatment with the SIRT3 shRNA plasmid abolished this effect ($P < .05$, compared with the DM+I/R+H group).

As shown in Figs 6 (*C* and *D*), the biochemical assay showed that DM injury to the lung induced the release of cytochrome c from the mitochondria ($P < .05$, compared with the Con+Sham group). I/R injury also induced the release of cytochrome c from the mitochondria ($P < .05$, compared with the Con+Sham group). Diabetic animals subjected to lung I/R showed a further increase in the release of cytochrome c from the mitochondria ($P < .05$, compared with the Con+I/R group). Similar increases were observed in the DM+I/R group ($P < .05$, compared with the DM+Sham group). Systemic administration of GYY4137 decreased the release of

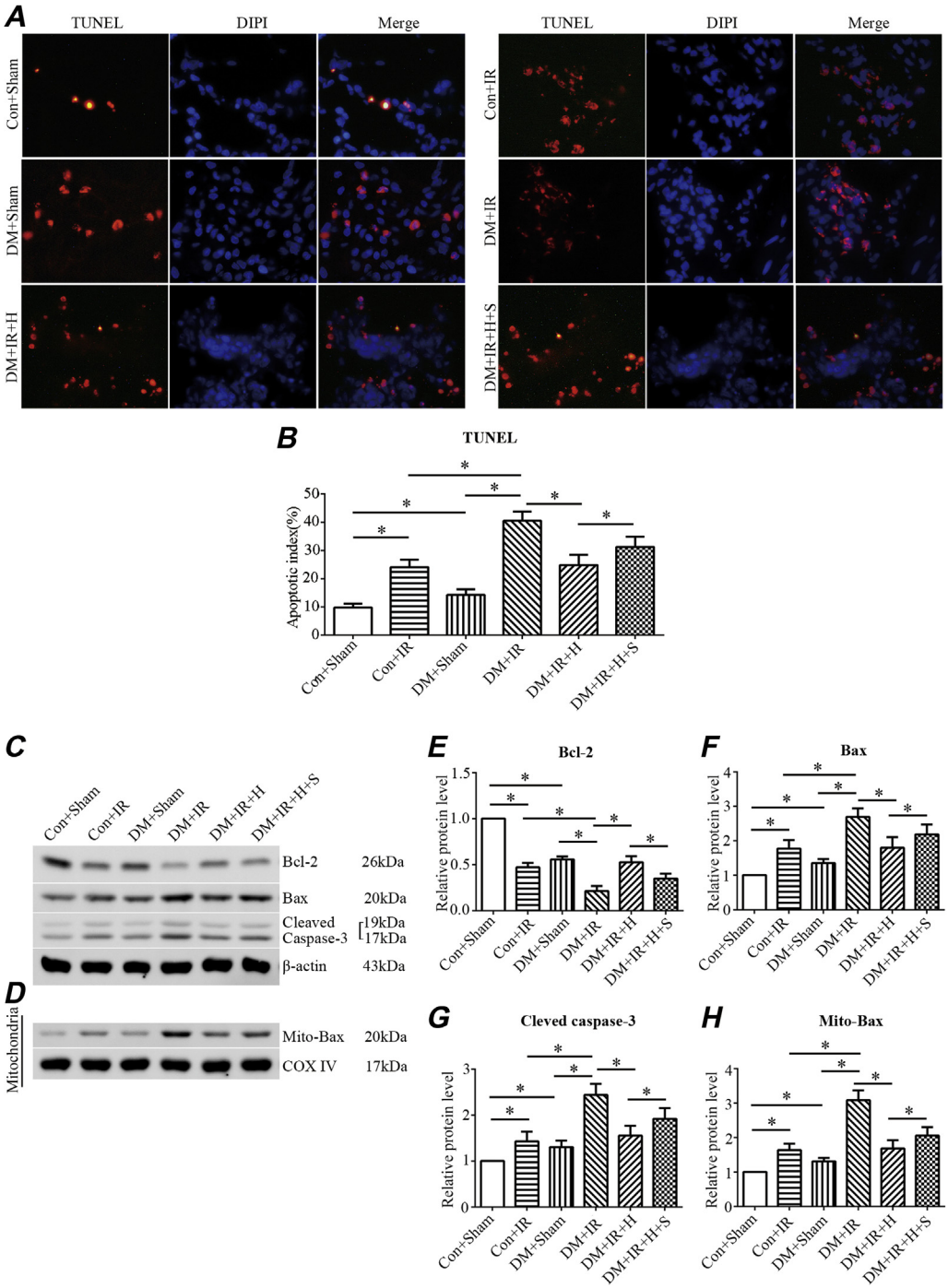

Fig 5. SIRT3 mediates the antiapoptotic effect of H_2S in diabetic lung I/R injury. (*A*) Representative in situ detection of lung parenchymal cell apoptosis by TUNEL staining (magnification: 400×). (*B*) Percentage of TUNEL-positive nuclei. (*C*) Representative blots. (*D*) Representative blots. (*E*) Bcl-2 expression. (*F*) Bax expression. (*G*) Cleaved caspase-3 expression. (*H*) Mitochondrial bax expression. (*$P < .05$ versus others; $n = 8$ in each group). TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end-labeling.

cytochrome c ($P < .05$, compared with the DM+I/R group), and the SIRT3 shRNA plasmid partially abolished this effect ($P < .05$, compared with the DM+I/R+H group).

We used JC-1, a fluorescent dye extremely sensitive to small changes in mitochondrial membrane potential ($\Delta\psi m$). As shown in Fig 6 (*E*), DM injury to the lung induced a depolarization of the mitochondrial membrane potential in the lung ($P < .05$, compared with the Con+Sham group). Similar depolarization was observed in the Con+I/R group ($P < .05$, compared with the Con+Sham group). Diabetic animals subjected to lung I/R showed even further mitochondrial depolarization ($P < .05$, compared with the Con+I/R group). Similar decreases in mitochondrial depolarization were observed in the DM+I/R group ($P < .05$, compared with the DM+ Sham group). The GYY4137 treatment attenuated mitochondrial depolarization ($P < .05$, compared with the DM+I/R group); whereas this effect was partially abolished by SIRT3 inhibition ($P < .05$, compared with the DM+I/R+H group).

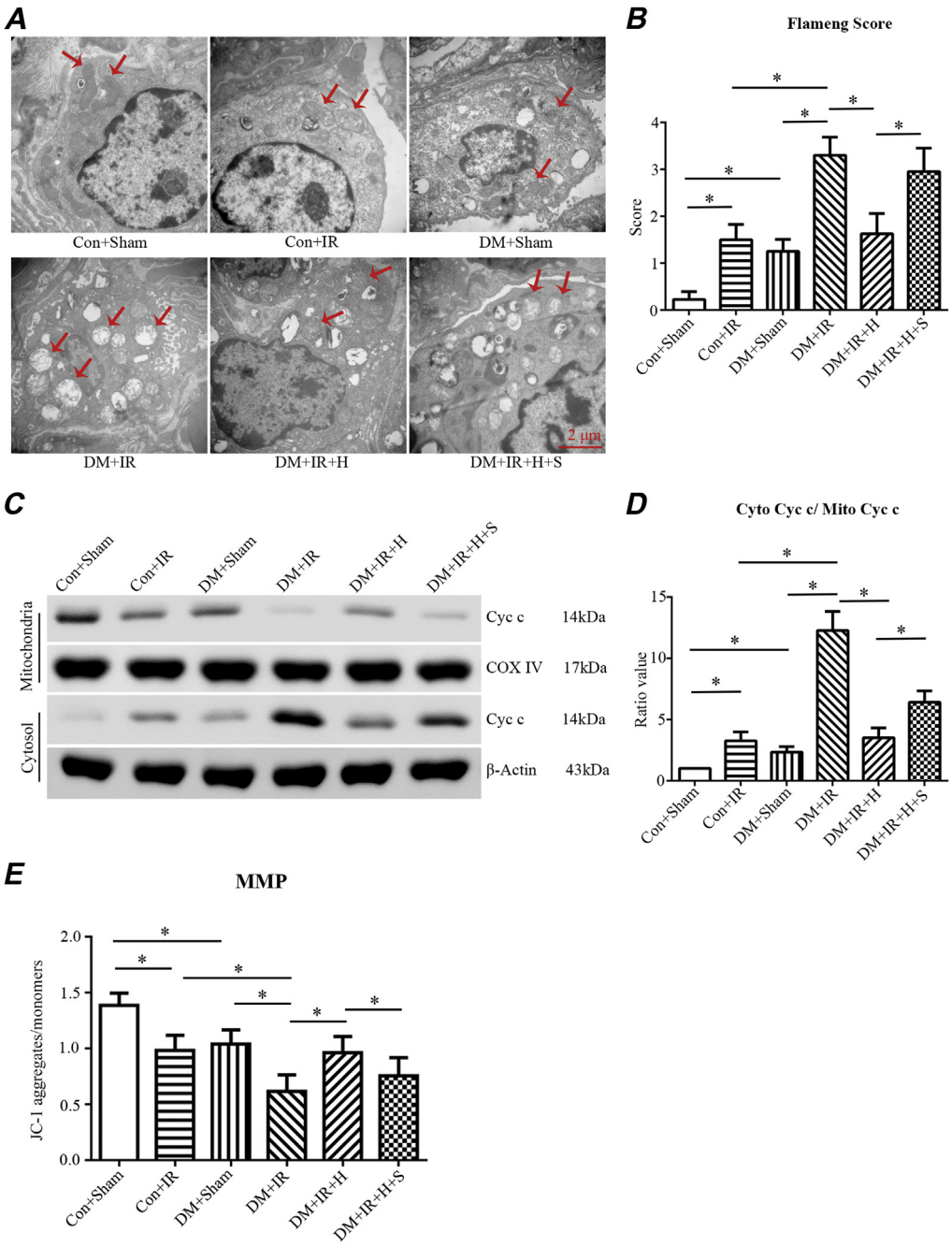

Fig 6. The SIRT3 signaling pathway participates in the protective effects of H_2S on mitochondrial function in diabetic lung I/R injuryin rats. (*A*) Mitochondrial ultrastructure. Mitochondria (*arrows*) in type II alveolar epithelial cells were imaged with transmission electron micrographs (magnification: 20,000×). (*B*) Flameng score. (*C*) Representative blots. (D) Cytochrome c in mitochondria and cytosol. (*E*) Determination of mitochondrial membrane potential. *Cyc c*, cytochrome c; *MMP*, mitochondrial membrane potential. ($^*P < .05$ versus others; $n = 8$ in each group)

Discussion

The salient findings of this study are as follows:

- Lung SIRT3 signaling was dramatically downregulated in animals with type 2 diabetes, and it was further decreased by the insult of I/R. H_2S administration via the H2S donor GYY4137effectively enhanced SIRT3 signaling and attenuated the lung I/R injury in type 2 diabetic rats.
- H_2S also alleviated oxidative stress, apoptosis, and inflammation, and in particular, preserved mitochondrial function by reactivating the SIRT3 signaling pathway, thus ameliorating lung I/R injury under type 2 diabetic conditions.

Emerging data have suggested that DM, especially type 2 DM, is associated with an increase in risk factors for lung disease.[2,3,30,31] In diabetic patients, the risk of an exaggerated I/R injury is more serious, and the long-term prognosis is less favorable than in the nondiabetic population.[32,33] Hyperglycemia upregulates poly ADP ribose polymerase (PARP), the polyol pathway and protein kinase C pathways, which can lead to a nicotinamide adenine

dinucleotide+hydrogen (NADH) and nicotinamide adenine dinucleotide$^+$ (NAD^+) redox imbalance with increased levels of NADH and decreased levels of NAD^+.[2,34] All these eventually contribute to the production of ROS that then induces oxidative stress, inflammation, mitochondrial dysfunction, and cell death, which exaggerate the I/R injury.[7] Because mitochondria are the primary source of ROS and a target of ROS, mitochondrial dysfunction may be a crucial factor contributing to I/R injury in the diabetic state.[6,7] We found that DM significantly worsened physiologic parameters (lung edema, pulmonary compliance, and oxygenation index) and decreased the survival rate after the period of I/R. We also observed increased oxidative stress, increases in apoptosis, and increases in markers of inflammation, and in particular, mitochondrial dysfunction, which closely reflects the severity of local lung injury. SIRT3 is recognized as the most important acetyl-lysine deacetylase that regulates many levels of mitochondrial function and ROS generation.[15] SIRT3 regulates ROS homeostasis by altering the acetylation of SOD2, the major enzymatic superoxide scavenger localized in the mitochondria.[15] Hyperglycemia contributes to the decrease of NAD^+ content, which implies that the levels of SIRT3 would be attenuated as well, given that protein expression of the Sirtuins is NAD^+-dependent.[15] Other studies have shown that the SIRT3 signaling is downregulated in the diabetic lung, which is consistent with the results of studies reporting that the SIRT3 signaling showed decreased expression in diabetic tissues.[2,21,35,36] In addition, decreased SIRT3 expression can lead to a greater level of I/R injury.[19] Upregulating the SIRT3 activity or expression also represents a potentially promising therapeutic strategy to ameliorate I/R-induced tissue damage.[18,20] Consistent with these studies, we observed markedly downregulated lung SIRT3 signaling in diabetic conditions, and reperfusion injury further decreased the SIRT3 signaling, thus aggravating I/R-induced oxidative damage. Enhanced oxidative stress further induced mitochondrial dysfunction, inflammation, and apoptosis. Together, the findings from our study strongly suggest an association between the mechanisms underlying the diabetic state aggravating lung I/R injury and impaired SIRT3 signaling. The molecular mechanisms mediating downregulation of SIRT3 during diabetic lung I/R, however, remains to be elucidated.

Another main finding of this study relates to the protective effect of H_2S against lung I/R injury via activating the SIRT3 signaling pathway in the setting of type 2 DM. There is clear evidence that systemic H_2S is decreased in diabetic animal models because hyperglycemic cells consume and oxidize H_2S.[10,37] Moreover, H_2S attenuates myocardial I/R injury through mitochondrial protection in animals with type 2 diabetes.[38] Intriguingly, accumulating evidence suggests that H_2S is able to upregulate the SIRT3 gene expression via enhancing activator protein 1 (AP-1) binding activity with the SIRT3 promoter.[22,24] The H_2S induces expression of nicotinamide phosphoribosyltransferase (NAMPT), which functions as the rate-limiting enzyme to convert nicotinamide (NAM) to nicotinamide mononucleotide (NMN). The NMN is then converted to NAD^+ via NAMPT.[39,40] The H_2S may participate in regulating the ratio of NAD^+-to-NADH to alleviate redox imbalance resulting from diabetes.[39] As a NAD^+-dependent deacetylase, the SIRT3 signaling may be enhanced by H_2S, which then upregulates NAD^+ levels.[15,39,41] The H_2S may also improve mitochondrial function by upregulating the SIRT3 expression, thus decreasing the oxidant-provoked cell dysfunction.[22] Thus, the relationship between H_2S and SIRT3 in lung I/R injury in diabetic rats attracts our attention. The results of our study demonstrated that the H_2S reversed the decrease in the SIRT3 expression in the lungs of rats with type 2 diabetes that were exposed to this model of I/R injury. We also found that the H_2S protected against lung I/R injury in rats with type 2 diabetes, and inhibition of the SIRT3 signaling attenuated the protective effect of the H_2S. Accordingly, our data suggests that H_2S protected against diabetic lung I/R injury and that SIRT3 played a pivotal role in this process.

Mitochondrial dysfunction also appeared to play a pivotal role in I/R-induced tissue injury in the diabetic state, and the resultant impaired mitochondrial integrity made cells susceptible to free radical generation and mitochondria-induced cellular apoptosis.[8,9] SIRT3 can alleviate mitochondrial injury induced by oxidative stress through its deacetylation function, which contributes to improved mitochondrial dynamics and the maintenance of mitochondrial function.[42,43] The amazing finding in our study is the electron microscopic findings that the mitochondrial injury was rescued by H_2S, whereas these protective effects were partially prevented by SIRT3 siRNA. We also found that the inhibition of the SIRT3 expression promoted the depolarization of the mitochondrial membrane potential, a global index of mitochondrial function. In contrast, H_2S counteracted this mitochondrial membrane depolarization to the level that $\Delta\psi m$ was normalized apparently via activating the SIRT3 signaling pathway. Similarly, there may be a functional role for SIRT3 in preserving mitochondrial integrity by preventing loss of the membrane potential.[43] Cytochrome c is a part of the electron transport chain localized in mitochondria. Once the integrity of the mitochondria is destroyed, cytochrome c can be released from the mitochondria, triggering caspase-3 activation and eventually leading to apoptosis.[44] By preventing mitochondrial dysfunction, SIRT3 may contribute to the inhibition of the mitochondrial cytochrome c release to the cytoplasm.[45,46] Our study showed that the H_2S alleviated the release of cytochrome c from mitochondria in diabetic lung I/R injury, and the SIRT3 deficiency increased the cytosolic accumulation of cytochrome c, presumably because of the release of cytochrome c from the mitochondria. In our experimental setting, the H_2S preserved mitochondrial function and limited the mitochondrial morphologic alterations, a protective effect that possibly is attributed to the activation of SIRT3 signaling, thus ameliorating lung I/R injury in the diabetic state.

Hyperglycemia enhances oxidative stress in the diabetic state that eventually aggravates I/R damage.[47] SIRT3 increased the acetylation of SOD2, a key antioxidant enzyme involved in scavenging superoxide radicals in the mitochondria and regulating ROS homeostasis.[15] Other studies have demonstrated that upregulating the SIRT3 expression or activity represents a promising therapeutic method of attenuating oxidative stress-induced tissue damage.[18,48,49] In this study, we observed that H_2S normalized the SIRT3 expression and decreased oxidative stress. The SIRT3 inhibition also markedly abolished the antioxidative effects of H_2S. SIRT3 exerts antiapoptotic effects during diabetic lung I/R injury in addition to its antioxidative activity. The SIRT3 overexpression activated NF-κB downstream target genes Bcl-2 and SOD2, increased the ratio of Bcl-2 to bax, and thus protected cells against bax-mediated apoptosis.[48] Under basal conditions, bax is kept in the cytoplasm by deacetylated Ku70, which prevents the translocation of the bax into the mitochondria, consequently preventing apoptosis.[50] A recent study showed that SIRT3 physically binds to and deacetylates Ku70, which promotes the interaction of Ku70 with the pro-apoptotic protein bax. Under conditions of stress, the SIRT3 overexpression prevented cell death by hindering the translocation of bax into the mitochondria.[51] Here, we found that the diabetic lung I/R-induced downregulation of SIRT3 was associated with the decreased expression level of antiapoptotic factors and the increased expression levels of proapoptotic factors. Notably, we also found that the mitochondrial distribution of bax was markedly

increased after diabetic lung I/R injury. The H_2S treatment ameliorated the apoptosis-related molecular changes by increasing the expression of SIRT3, and SIRT3 inhibition diminished the antiapoptotic effects of the H_2S. These data suggest that SIRT3-mediated alleviating of oxidative stress and apoptosis played a key role in the protective actions of H_2S in diabetic lung I/R injury.

Several limitations of this study should be noted. First, we developed a high-fat diet–fed streptozotocin-induced model of type 2 diabetes in the rat, which may not be representative of the underlying mechanism in type 2 DM in humans. This model, however, is well recognized in the screening of antidiabetic drugs.[25] We used this model to simulate the clinical presentation of type 2 DM patients, but whether this model is truly representative of diabetes requires further detailed elucidation. Second, we did not investigate the effect of the H_2S treatment in nondiabetic animals after lung I/R injury in this study. We confirmed elsewhere that H_2S attenuated lung I/R injury by decreasing oxidative damage and ameliorating mitochondrial injury.[14] Additional studies are needed to investigate the potential roles of the SIRT3 signaling in the H_2S treatment in nondiabetic rats.

In summary, the present study shows that the H_2S treatment protected against lung I/R injury by alleviating oxidative stress, apoptosis, inflammation, and especially by preserving mitochondrial function via SIRT3 reactivation in rats with type 2 diabetes. Exogenous H_2S protection may have translational value for treating acute lung injury induced by I/R injury in patients with type 2 DM.

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