

Isoflurane attenuates LPS-induced acute lung injury by targeting miR-155-HIF1-alpha

Rong Hu¹, Ying Zhang¹, Xiaohua Yang², Jia Yan¹, Yu Sun¹, Zhifeng Chen¹, Hong Jiang¹

¹Department of Anesthesiology, Shanghai Ninth People's Hospital affiliated to Shanghai Jiao Tong University, School of Medicine, No. 639, Zhi Zao Ju Road, Shanghai, China, ²Central Laboratory, Shanghai Chest Hospital affiliated to Shanghai Jiao Tong University

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1. ABSTRACT

Isoflurane alleviates the inflammatory response in endotoxin-induced acute lung injury (ALI). In this study, we investigated the protective mechanism of isoflurane postconditioning in lipopolysaccharide (LPS)-induced ALI. Exposure to isoflurane decreased miR-155 and upregulated HIF-1 alpha and HO-1 mRNA and protein. The effects of isoflurane on HIF-1 alpha mRNA and protein could be inhibited by overexpression of miR-155. Furthermore, mice overexpressing miR-155 had higher levels of TNF-alpha and IL-1 beta in BALF when exposed to isoflurane after LPS challenge.

Conversely, downregulation of miR-155 promoted isoflurane effects on HIF-1 alpha expression. These results suggest that isoflurane posttreatment at 1 MAC for 4 hour alleviates LPS-induced ALI and cell injury by triggering miR-155-HIF-1 alpha pathway, leading to upregulation of HO-1.

2. INTRODUCTION

Endotoxemia-induced acute lung injury (ALI) and its severe form, acute respiratory distress syndrome, are major causes of death in intensive

care units (1). Pathological changes associated with ALI are intense inflammation accompanied by disruption of the epithelial integrity, interstitial edema, and accumulation of neutrophils (2, 3). The mechanism of endotoxin-induced ALI and an effective therapeutic regimen are still unknown.

Excessive inflammation is avoided through regulation by critical factors (4). Hypoxia inducible factor 1 α (HIF-1 α) is a transcription factor that triggers cytoprotection and metabolic alterations in response to hypoxia (5, 6). HIF-1 α induces the transcription of over 40 genes in oxygen homeostasis to diminish oxygen tension; one of the targeted genes is heme oxygenase-1 (HO-1) (7, 8). HO-1 has been recognized to exhibit important immune modulatory and anti-inflammatory functions (9). Hypoxia is connected to inflammation and studies show that HIF-1 α is crucial for innate and adaptive immunity, particularly in cellular metabolism and oxygen delivery (10, 11). Stabilization of HIF-1 α and induction of its downstream genes have anti-inflammatory effects (12, 13).

MicroRNAs (miRNAs) are small, noncoding RNAs of approximately 22 nucleotides that regulate gene expression through translational suppression or mRNA degradation. MiRNAs recognize target sites in 3'-untranslated regions (UTRs) with specific but imperfect pairing (14, 15). Recently, miR-155 was shown to be critical for control of innate immunity. MiR-155 is highly induced in macrophages stimulated with toll like receptor (TLR) ligands such as poly (I: C) and lipopolysaccharide (LPS) (16). Overproduction of miR-155 during macrophage activation promotes and sustains inflammation (17, 18). In prolonged hypoxia, HIF-1 α is a target of miR-155, with miR-155 triggering the resolution of HIF-1 α (19). Our preliminary investigation identified a sequence in the HIF-1 α 3'-UTR that was highly consistent with the target sequence for the seed region of miR-155. Therefore, we hypothesized that suppression of miR-155 might derepress HIF-1 α expression to attenuate the inflammatory response.

Isoflurane, a halogenated ether, is a potent inhalation agent in surgery and a sedative used in intensive care units (20). Growing evidence shows that isoflurane has anti-inflammatory properties. It is reported to prevent cytokine secretion from macrophages after LPS challenge (21, 22). *In vivo* studies showed that isoflurane protects against sepsis-induced mortality, reduces recruitment of polymorphonuclear leukocytes, decreases

protein leakage in the microvasculature, and inhibits cytokine release in lung tissue (23). In our previous study, we demonstrated that isoflurane preconditioning resulted in potent pulmonary protection. Posttreatment with isoflurane attenuates ALI by improving vascular permeability through overexpression of HO-1 (24). However, the molecular basis and the anti-inflammatory mechanism of isoflurane effects are still poorly understood. In this study, we provide evidence that posttreatment of cells with isoflurane limits inflammatory reactions by triggering a miR-155-HIF-1 α pathway, leading to upregulation of HO-1.

3. MATERIALS AND METHODS

3.1. Cell culture and treatment

The murine alveolar macrophage cell line MH-S was obtained from the cell repository of Shanghai Institute for Biological Science (Shanghai, China), and the mouse lung epithelial type II cell line MLE-12 was obtained from ATCC (Manassas, VA). MH-S cells were grown in 1640 medium with 10% fetal bovine serum (FBS) (Gibco), while MLE-12 cells were cultured in HITES medium with 2% FBS (Gibco) (25). Cell medium were supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin.

To determine whether posttreatment of cells with isoflurane alleviated cell injury, MH-S and MLE-12 cells were stimulated with 100 μ g/ml LPS for 24 h with or without posttreatment with isoflurane (26). The indicated cells were incubated with isoflurane (0.1.6., 0.3.2. or 0.6.4. mM; Abbott Laboratories, Abbott Park, IL) for 0 h, 2 h, 4 h, or 8 h in sealed chambers inside incubators as described previously (27). The cells without any treatment were served as normal control.

3.2. CCK8 assay

MH-S and MLE-12 cells in logarithmic growth were digested with trypsin to single suspensions and 8000 to 10,000 cells were transferred to 96-well plates for treatment as indicated. CCK-8 (cell counting kit-8, Dojindo, Japan) was added into the 96-well plates. OD (optical density) at 450 nm was read with a microplate reader 2 to 4 h after incubating with reagent. Each experiment was performed 3 times independently.

3.3. Plasmid construction

To construct psiCHECK-HIF-1 α -3'UTR-WT (wild type, WT), the 3'-UTR fragment of HIF-1 α (Genebank accession: NM_010431) containing

binding sites of miR-155 (from 2935 to 4775 bp in the HIF-1 α mRNA), was amplified by PCR using primers 5'-AACCCCTCGAGGCGTTTCTAATCTCATT-3' and 5'-AACCGCGGCCG CAAGCTGGAAGGTTTGTG-3', and cloned into *Xho*I/*Not*I sites of psiCHECK-2 (Gifted by Dr. Cheng-You Jia, from central laboratory of Shanghai Tenth People's Hospital of Tongji University) for simultaneous expression of firefly and renilla luciferases. The HIF-1 α 3'-UTR was cloned downstream of the *Renilla* gene in a modified psiCHECK-Control vector. Firefly luciferase control reporter serves as the control reporter.

PsiCHECK-HIF-1 α -3'-UTR-mut (mutated) was generated as the same method above by mutating four nucleotides of the HIF-1 α 3'-UTR in the miR-155 seed sequence at position 3971 to 3974bp, change GCAT to CGTA (Figure 4A). PCR primers were: 5'-ATTCTAGAGCTAGCGTTCATAATCGTTCACTCTAC-3' and 5'-TCCTTCGCGGCCGCGAGTTGAGATGTTGTTTAGG-3'. Mutations were confirmed by DNA sequencing.

3.4. Luciferase reporter assays

MH-S cells were transfected with 50 nM RNA oligonucleotides that were mmu-miR-155 mimics (5'-UUAUUGC UAAUUGUGAUAGGGGU-3') or mmu-miR-155 inhibitor (5'-ACCCCUAUCA CAAUUGACAUUAA-3') (Genepharma, Shanghai) with psiCHECK-2 HIF-1 α 3'-UTR plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. MiRNA mimics control (5'-AGTGTGAGTTCTACCATTGCCAAA-3'), miRNA inhibitor control (5'-CAGUACUU UUGUGUAGUACAA-3') and psiCHECK-2-HIF-1 α 3'-UTR-mut served as controls. After transfection for 48 h, cells were washed with PBS twice and lysed with 100 μ l passive lysis buffer (Promega E194A, Madison). Dual Luciferase Reporter Assay kits (Promega, Madison, WI) were used to measure luciferase activity using an illuminometer (Lumat LB 9507, Berthold, Germany). Experiments were repeated 3 times independently.

3.5. Transfection with HIF-1 α siRNA

RNA interference was performed with HIF-1 α siRNA or negative control siRNA (Genepharma, Shanghai). Lipofectamine 2000 (Invitrogen) was used for cell transfection according to the manufacturer's instruction. MH-S and MLE-12 cells were transfected with 50 nM siRNA at 60% confluence. After transfection for 6 h, normal growth medium was replaced. After incubation for 18 h, the cells were stimulated with

100 μ g/ml LPS for 24 h, or followed with 0.32 mM isoflurane for 4 h. HIF-1 α siRNA was HIF-1 α -MUS-606: 5'-GCUCACCAUCAGUUAUUUATT-3', and the negative control siRNA was 5'-UUCUCCGAACGUGUCACGUTT-3'.

3.6. Gene transfection with miR-155, miR-155 EV, miR-155 inhibitor, and miR-155 inhibitor EV

The lentivirus-mediated miR-155 packaging system contained three plasmids: GV217-miR-155 (or GV217 empty vector (EV)), pHelper 1.0., and pHelper 2.0. at 4:3:2. The plasmids in the lentivirus-mediated miR-155 inhibitor packaging system were GV159-miR-155 inhibitor (or GV159 EV), pHelper 1.0., and pHelper 2.0. at 4:3:2. Plasmids (12 μ g) were co-transfected with 30 μ l Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) into HEK-293T cells, and cultured in 10 cm diameter culture dishes. Supernatants were collected after 48 h infection and filtered with 0.45 micrometer pore filters and used as a source of virus. The final viral titer was 8×10^8 titre unit (TU)/ml for mmu-miR-155 and mmu-miR-155 EV, and 1×10^9 TU/ml for mmu-miR-155 inhibitor and mmu-miR-155 inhibitor EV.

MH-S and MLE-12 cells were infected with miR-155, miR-155 EV, miR-155 inhibitor, and miR-155 inhibitor EV with 5 μ g/ml polybrene (Sigma) for 12 h, and medium was refreshed. After infection for 96 h, efficiency was evaluated with a fluorescent microscope. Cells were sorted using fluorescent activated cells sorter (FACS; Becton Dickinson, Mountain View, CA, USA) according to the GFP expression from GV217 and GV159 plasmids. Sorted cells were used for experiments. Lentiviruses (2.5×10^7 TU in 200 μ l) were injected into mice through the caudal vein 14 days before experiments.

3.7. Animals and experimental protocols

As described previously, isoflurane was delivered via a custom-built chamber that allowed isoflurane to flow in and out of opposite ends of the chamber (28). Mice inhaled 1.4% isoflurane, which was 1 minimal alveolar concentration (MAC) for mice, balanced with air through an isoflurane-specific vaporizer (Datex-Ohmeda Inc., Madison) for 4 h. Negative and positive control animals, which received saline or LPS, were exposed to air (29). Isoflurane and oxygen concentrations were monitored by Datex infrared analyzer (Capnomac, Helsinki, Finland) from a hole near the outlet. The concentration of inhaled anesthetic used in this study

did not cause any cardiorespiratory complications in preliminary experiments.

Male C57BL/6 mice at 6–8 weeks of age used in our study were obtained from SLAC National Rodent Laboratory Animal Resource (Shanghai, China). All animals were approved by the Institutional Animal Care and Use Committee of the Ninth People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine. Mice were housed in a laminar flow cabinet with a 12 h light/12 h dark cycle with free access to food and water. One hundred and sixty mice were randomized into five groups ($n = 32$ per group), which injected via tail veins with 2.5×10^7 TU miR-155, miR-155 inhibitor, miR-155-EV, or miR-155 inhibitor-EV lentiviruses respectively (30). And mice of one group without injection of lentiviruses were used as controls (Non-injected). 14 days after injection, mice in each group were divided into four groups ($n = 8$ per group): (1) Control group (Ctrl): mice injected with saline and inhaled air; (2) Isoflurane group (ISO): mice injected with saline and postconditioning with 1.4% isoflurane for 4 h; (3) LPS group (LPS): mice posttreated with air for 4 h after receiving LPS (O111:B5, Sigma L2880; 30 mg/kg intraperitoneal injection) for 24 h (31); (4) LPS-Isoflurane group (LPS+ISO): animals inhaled 1.4% isoflurane for 4 h after LPS stimulation for 24 h. At the end of treatment in each group, mice were sacrificed by decapitation, bronchoalveolar lavage was carried out to collect bronchoalveolar lavage fluid (BALF) for cytokine assays, and their lungs were harvested for histological analysis or molecular analysis.

3.8. Quantitative real-time PCR (Q RT-PCR)

Total RNA from cells or lung tissue were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). Single-stranded cDNAs were reverse synthesized using EasyScript cDNA synthesis kits (Applied Biological Materials), and miRNA was analyzed using TaqMan MicroRNA Reverse Transcription kits (Applied Biosystems). Q RT-PCR was performed in an ABI Prism 7900HT sequence detection system (Applied Biosystems), using KAPA SYBR FAST qPCR kit Master MIX (Lifetech, Carlsbad, CA) for miR-155 quantification and SYBR green PCR reagent kits (Clontech) to quantify HIF-1 α transcription. The primers for Q RT-PCR were as follows: miR-155, forward: 5'-GCCCGTTAATGCTAATTG-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'; U6, forward: 5'-CTCGCTTCGGCAGCAC-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; HIF-1 α , forward: 5'-TAAATGTTCTGCCACCCT-3', reverse:

5'-GCGACAAAGTGCATAAAACC-3'; β -actin, forward: 5'-GAGCTACG AGCTGCC TGACG-3', reverse: 5'-CCTAGAAGCATT TCGGGTGG-3'. snRNA U6 was used as an internal control for miR-155 and β -actin for HIF-1 α .

3.9. Western blot analysis

Total protein was extracted from cultured cells or mouse lung tissue using RIPA lysis buffer (50 mM Tris-HCl pH7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1 mg/ml leupeptin, 1 mg/ml aprotinin and 2.5 mM sodium orthovanadate). Briefly, 50–80 μ g samples were separated by SDS-PAGE and transferred to PVDF membranes. Western blot was carried out as described previously (28). Antibodies were HIF-1 α (1/500, ab113642, Abcam), HO-1 (1/1000, ab85309, Abcam) and β -actin (1/1000, A2066, rabbit polyclonal; Sigma). Bands were visualized using an enhanced chemiluminescence reagent kit (Amersham Life Science, Cleveland, OH). The densitometric values were determined using a gel image analysis system (Bio-Rad, Hercules, CA) normalized to β -actin.

3.10. Histopathology

Mice were decapitated and lung tissue was harvested for histopathology. Pulmonary changes in morphology were determined 24 h after stimulation with LPS, with or without isoflurane. Lung tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 μ m at room temperature. Samples were stained with hematoxylin and eosin (H&E) and examined by light microscopy. The degree of lung injury was evaluated using a scoring system based on predetermined criteria (32) (Table 1). Two independent investigators read slides in a blind manner and the average scores were determined. Injury score was determined according to the formula: ((alveolar hemorrhage points/number of fields) + 2 \times (alveolar infiltrate points/number of fields) + 3 \times (fibrin points/number of fields) + (alveolar septal congestion/number of fields))/total number of alveoli counted.

3.11. IL-1 β and TNF- α level in bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was collected by irrigating left lungs twice with 2 ml saline at the end of experiments. After centrifuging at 200 $\times g$ for 10 min, IL-1 β and TNF- α levels in BALF were determined using an ELISA kit (R&D Systems Inc., Minneapolis, MN, USA).

Table 1. Semiquantitative histopathology score of lung injury

Tissue	0	1	2	3
Alveolar Septae	All septae are thin and delicate	Congested alveolar septae in less than 1/3 of the field	Congested alveolar septae in 1/3 to 2/3 of the field	Congested alveolar septae in more than 2/3 of the field
Alveolar Hemorrhage	No hemorrhage	At least 5 erythrocytes per alveolus in 1-5 alveoli	At least 5 erythrocytes per alveolus in 5-10 alveoli	At least 5 erythrocytes per alveolus in more than 10 alveoli
Intra-alveolar Fibrin	No intra-alveolar fibrin	Fibrin strands in less than 1/3 of the field	Fibrin strands in 1/3 to 2/3 of the field	Fibrin strands in greater than 2/3 of the field
Intra Alveolar Infiltrates	Less than 5 intra-alveolar cells per field	5-10 intra-alveolar cells per field	10-20 intra-alveolar cells per field	More than 20 intra-alveolar cells per field

3.12. Immunohistochemistry

Lung tissue sections (4 μ m) were dewaxed in xylene, rehydrated with graded ethanol, rinsed in PBS three times for 5 minutes, and immersed in 3% hydrogen peroxide in 80% methanol for 10 min to block endogenous peroxidase activity. Slides were rinsed in PBS twice for 5 min, blocked with 5% bovine serum at 25°C for 20 min and incubated with mice monoclonal anti-HIF-1 α (Abcam, ab113642, UK; 1:200 dilution) at 4°C overnight. Mice IgG isotype was used as a negative control. Slides were incubated with biotinylated rabbit anti-mouse IgG secondary antibody, and incubated with H₂O₂-diaminobenzidine for 1 min. Samples were counterstained with hematoxylin.

3.13. Statistical analysis

All results were means \pm standard deviation (SD) of at least three independent experiments. Statistical analyses were calculated by Student's *t*-test or one-way ANOVA and *p* < 0.05 was considered significant. GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical calculations.

4. RESULTS

4.1. Isoflurane alleviates LPS-induced cell injury

We investigated the effects of isoflurane postconditioning in different concentrations on the viability of LPS-treated cells. MH-S and MLE-12 were stimulated with 100 μ g/ml LPS for 24 h with posttreatment of 0.16 mM, 0.32 mM, or 0.64 mM isoflurane for 4 h. It was found that the cell viability increased with the concentrations of isoflurane treatment and reached the highest value at 0.32 mM (Figure 1A, 1B). Therefore, 0.32 mM isoflurane was used in the following experiments. To find out the protective effects of isoflurane postconditioning

duration against LPS induced cell injury. MH-S and MLE-12 were stimulated with 100 μ g/ml LPS for 24 h with posttreatment of 0.32 mM isoflurane for 2 h, 4 h, or 8 h. It was found that the viability of cells posttreated with isoflurane (4 h or 8 h) was significantly higher than that in the LPS-treated cells without isoflurane post conditioning (*p*<0.05.) (Figure 1C, 1D). These data implied that postconditioning with 0.32 mM isoflurane for 4 h and 8 h have a protective effect on LPS-induced cells. In our study, the further investigations focused on posttreatment with 0.32 mM isoflurane for 4 h.

4.2. miR-155 and HIF-1 α are involved with the isoflurane effect on LPS-induced cell injury

To understand the potential mechanisms involved in the protective effects of isoflurane postconditioning against LPS-induced cell injury, we investigated the effects of isoflurane postconditioning on the levels of miR-155 and HIF-1 α in MH-S and MLE-12 cells. We exposed cells to 0.32 mM isoflurane for 4 h after challenging with LPS for 24 h. The untreated cells were served as control. HIF-1 α mRNA and miR-155 levels in MH-S and MLE-12 cells were determined by Q RT-PCR and the protein levels of HIF-1 α were detected by western blotting. LPS treatment significantly upregulated the levels of miR-155 (Figure 2A) and the expression of HIF-1 α both in mRNA and protein (Figure 2B, 2C) compared with the untreated control cells. However, postconditioning with isoflurane significantly downregulated the levels of miR-155 and upregulated both mRNA and protein levels of HIF-1 α in LPS-challenged cells compared with the LPS treatment cells without posttreatment with isoflurane (Figure 2). To further examine whether isoflurane reduced cell LPS-induced injury through HIF-1 α activation, MH-S and MLE-12 cells were subjected to siRNA mediated silencing of HIF-1 α (Figure 3A). CCK8 assay and western blots showed that silencing of HIF-1 α significantly reduced the cell viability (Figure 3B, 3C) and downregulated the

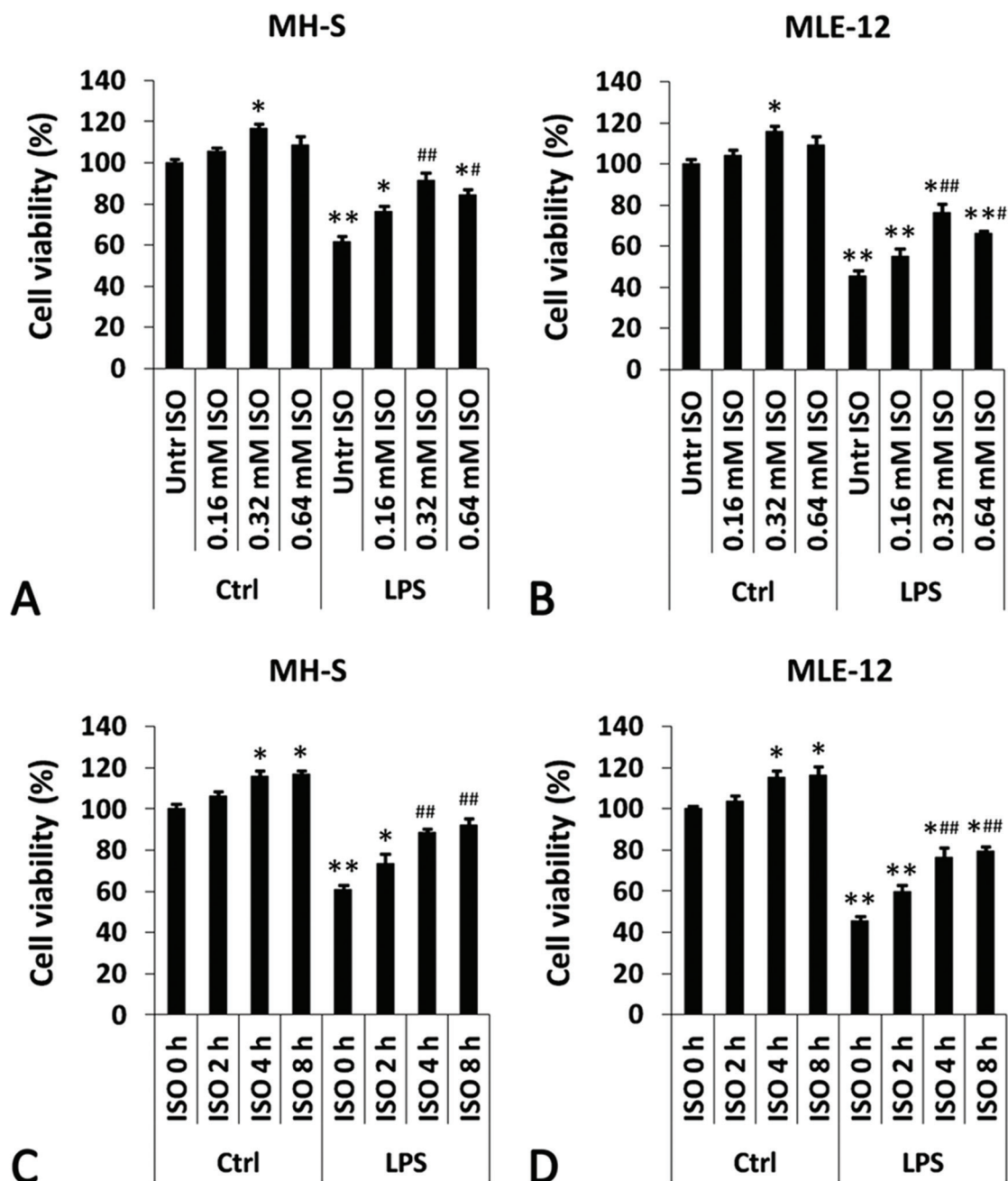


Figure 1. Isoflurane improved cell viability. (A-B) MH-S (A) and MLE-12 (B) were incubated with isoflurane (0.16 mM, 0.32 mM, or 0.64 mM) for 4 h, or stimulated with 100 μ g/ml LPS for 24 h, followed with 0.16 mM, 0.32 mM, or 0.64 mM isoflurane for 4 h. Cell viability was assessed by CCK8 assay. * p <0.05, ** p <0.01 compared with no ISO treatment cells (Untr ISO) of Ctrl group. # p <0.05 compared with Untr ISO of LPS group. (C-D) MH-S (C) and MLE-12 (D) were incubated with 0.32 mM isoflurane for indicated time periods (0 h, 2 h, 4 h, or 8 h) or stimulated with 100 μ g/ml LPS for 24 h, followed with 0.32 mM isoflurane for 0 h, 2 h, 4 h, or 8 h. Cell viability was assessed by CCK8 assay. * p <0.05, ** p <0.01 compared with ISO treated for 0 h (ISO 0 h) of Ctrl group. # p <0.05 compared with ISO 0 h of LPS group. Cells without LPS treatment were used as control group (Ctrl), and cells treated with LPS were served as injury group (LPS). Isoflurane was shorthand for ISO.

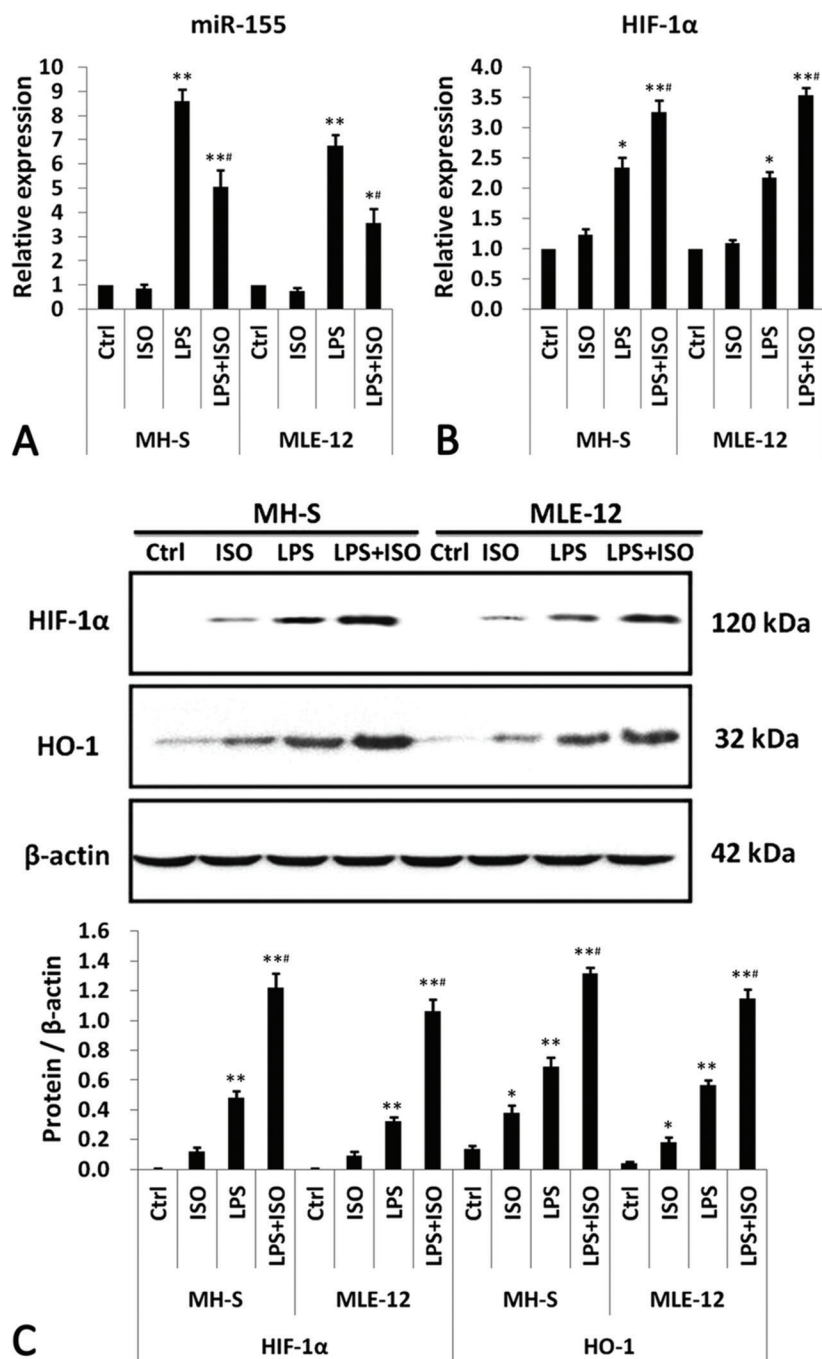


Figure 2. Effects of isoflurane on the expression of miR-155, HIF-1 α , and HO-1 *in vitro*. Cultured MH-S and MLE-12 were incubated with 0.32 mM isoflurane for 4 h, or stimulated with 100 μ g/ml LPS for 24 h, followed with 0.32 mM isoflurane for 4 h. Untreated cells were used as normal control (Ctrl), and cells treated with LPS only were used as injury control (LPS). (A) miR-155 and (B) HIF-1 α mRNA expression were detected by Q RT-PCR, normalized to the expression of snRNA U6 or β -actin and depicted as fold changes relative to the Ctrl group of each cell line. (C) Protein levels of HIF-1 α and HO-1 were detected by western blot. β -actin was used as the loading control. Quantification of HIF-1 α and HO-1 protein levels were performed by densitometry and normalized to β -actin levels. * p <0.05, ** p <0.01 compared with Ctrl group of each cell line. # p <0.05 compared with LPS group of each cell line.

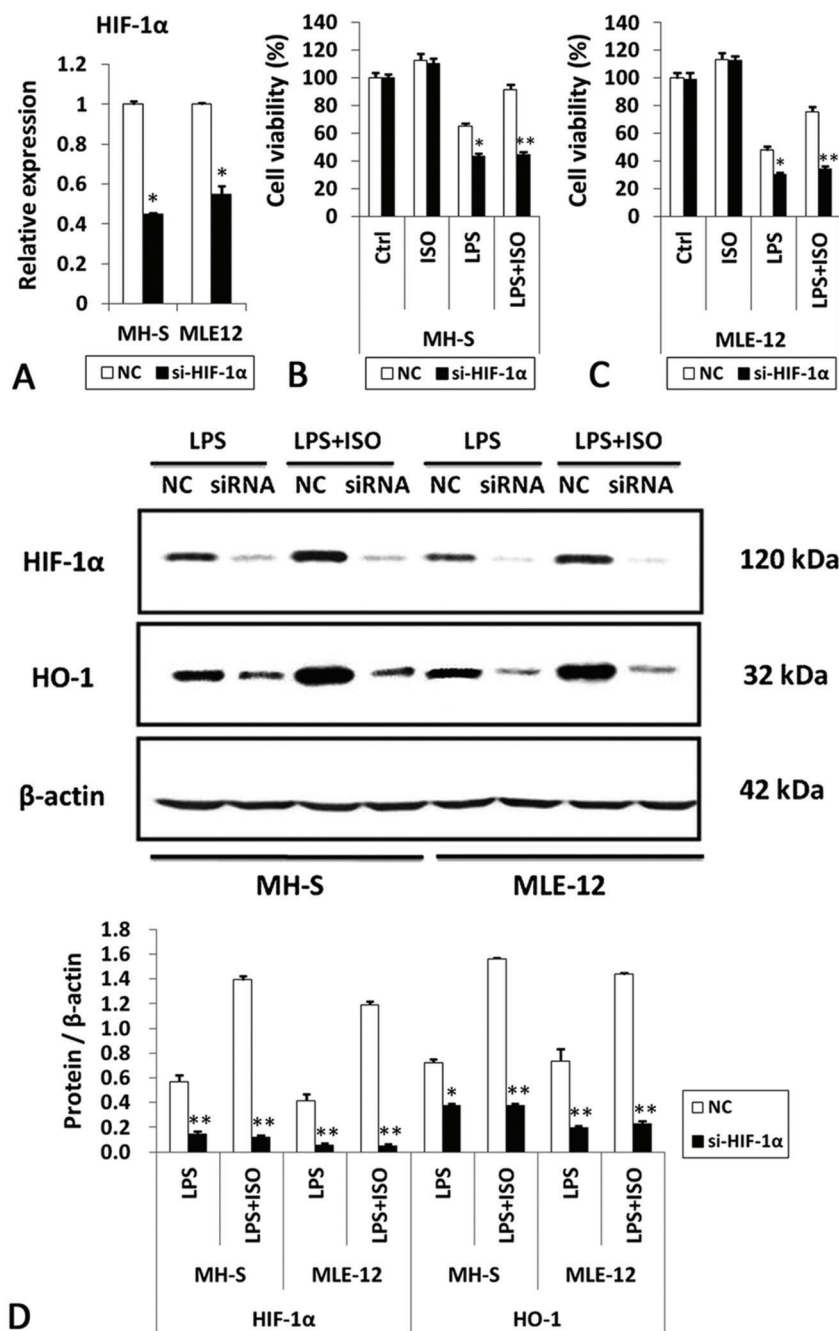


Figure 3. Isoflurane reduced cell LPS-induced injury through HIF-1 α activation. The HIF-1 α gene was knocked-down in MH-S and MLE-12 cells with transfection of siRNA against HIF-1 α (si-HIF-1 α), and nonsense siRNA transfection was treated as negative control (NC). (A) HIF-1 α mRNA was detected by Q RT-PCR, normalized to the expression of β -actin and depicted as fold changes relative to the corresponding NC group. * p <0.05 compared with NC group of each cell line. (B-C) The transfected cells were treated with 0.32 mM isoflurane for 4 h, or stimulated with LPS as indicated, followed with 0.32 mM isoflurane for 4 h. Cell viability of MH-S (B) and MLE-12 (C) was assessed by CCK8 assay. * p <0.05 compared with NC group in the same treatment group. (D) The transfected cells were treated with LPS for 24 h. Protein levels of HIF-1 α and HO-1 were detected by western blot, normalized to the levels of β -actin. * p <0.05 compared with NC group in the same treatment group of each cell line.

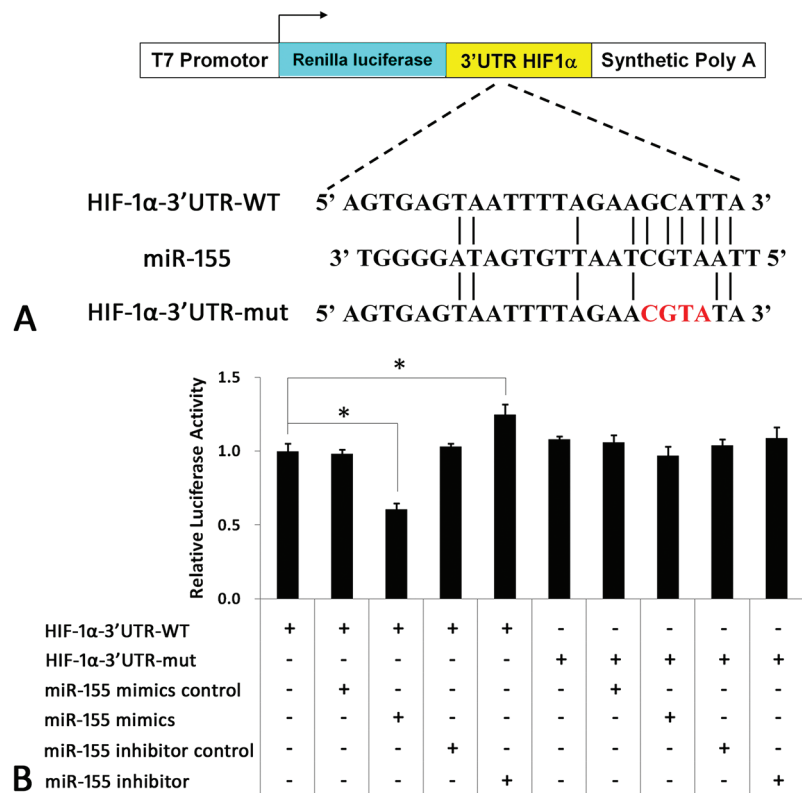


Figure 4. HIF-1α is a target gene of miR-155. (A) The top panel showed the structure and cloning site of psiCHECK™-2 vector. Wild (WT) or four nucleotides mutated (mut) within HIF-1α 3'-UTR was cloned into the reporter vector based on the predicted binding site in the 3'-UTR of HIF-1α with miR-155 seed sequence. (B) Dual luciferase assay of MH-S transfected with fluorescent reporter vectors containing either WT (blank control) or mut binding sites and miR-155 mimics or negative miRNA control mimics (miR-155 mimics control) or miR-155 inhibitor or negative miRNA control inhibitor (miR-155 inhibitor control). Luciferase activities were measured and normalized to the blank control. Expression of HIF-1α reporter was suppressed by miR-155 but not the mutated structure. * $p < 0.05$ compared with cells transfected with fluorescent reporter vectors containing 3'-UTR WT of HIF-1α.

protein levels of HO-1 (Figure 3D) of LPS-challenged cells with or without isoflurane treatment. The results suggest the involvement of the expression of miR-155 and HIF-1α in isoflurane treatment response to LPS-induced cell injury.

4.3. MiR-155 targets the HIF-1α gene

To confirm that the HIF-1α gene is a direct target of miR-155, luciferase reporter assays were performed in MH-S cells. HIF-1α wild-type (WT) or four nucleotides mutated (mut) within HIF-1α 3'UTR was cloned into a luciferase reporter vector based on the predicted binding site in the 3'UTR of HIF-1α with miR-155 seed sequence (Figure 4A), and co-transfected with miR-155 mimics or negative miRNA control mimics (miR-155 mimics control) or miR-155 inhibitor or negative miRNA control inhibitor (miR-155 inhibitor control) into MH-S cells. Luciferase reporter assays showed that miR-155 mimics

significantly decreased the luciferase activity of the 3'UTR-WT but not that of the 3'UTR-mut of HIF-1α in MH-S. Meanwhile, inhibition of miR-155 increased the luciferase activity after the co-transfection of miR-155 inhibitor and the 3'UTR-WT of HIF-1α into cells (Figure 4B). These results demonstrated that HIF-1α is a potential target of miR-155 and can be directly regulated by miR-155.

4.4. MiR-155 negatively regulates the expression of HIF-1α

To further investigate whether isoflurane exerts its function by modulating miR-155 and its target gene HIF-1α in LPS-induced cell injury, MH-S and MLE-12 cells were infected with lentivirus expressing or inhibiting miR-155. Overexpression of miR-155 significantly downregulated HIF-1α expression, whereas inhibition of miR-155 significantly upregulated the expression of HIF-1α in

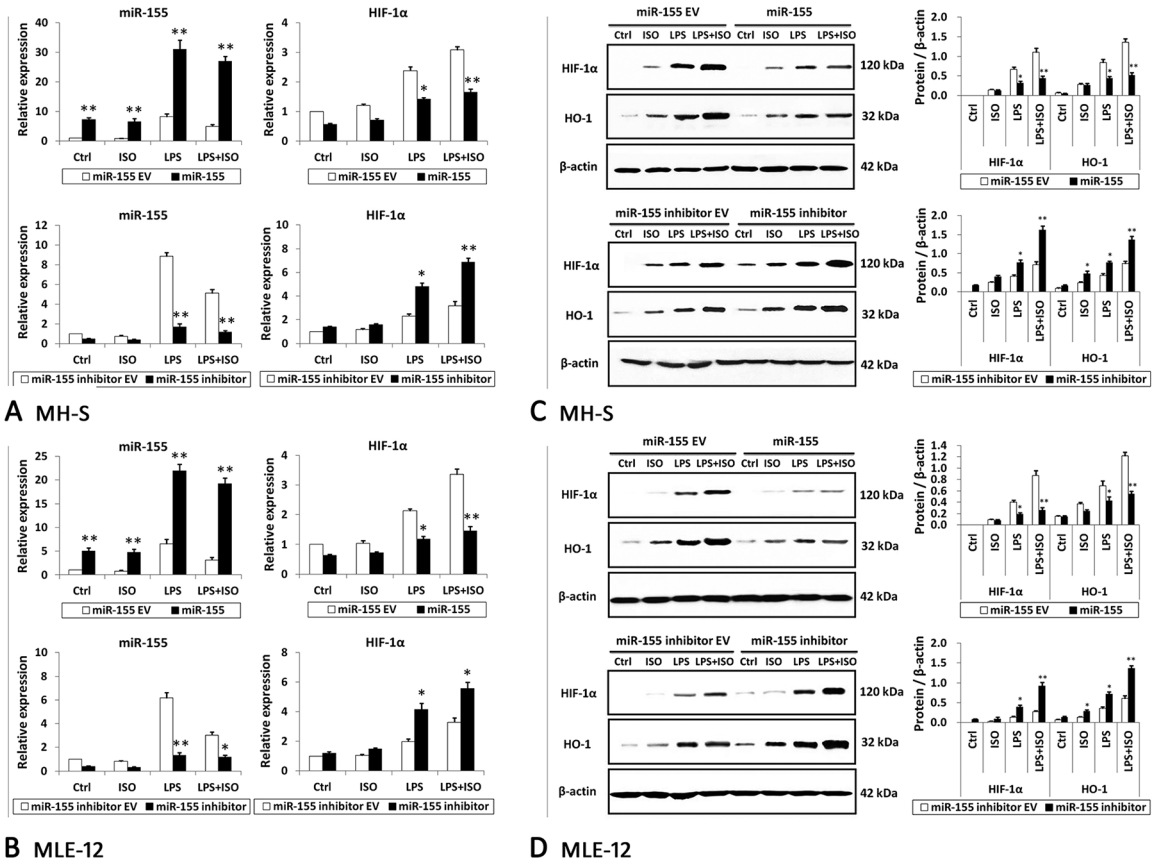


Figure 5. The expression of HIF-1 α and HO-1 is affected by miR-155 *in vitro*. MH-S or MLE-12 were infected with lentivirus expressing miR-155 (miR-155) or the GV217 empty vector control lentivirus (miR-155 EV), or lentivirus mediated miR-155 inhibitor (miR-155 inhibitor) or the GV159 empty vector control lentivirus (miR-155 inhibitor EV). After infection for 96 h, the infected cells were treated with or without isoflurane after LPS stimulation as described above. (A-B) The levels of HIF-1 α mRNA and miR-155 of MH-S (A) and MLE-12 (B) were measured by Q RT-PCR with β -actin or U6 level as an internal control. (C-D) The HIF-1 α and HO-1 protein levels of MH-S (C) and MLE-12 (D) were assessed by western blotting, normalized to the levels of β -actin. * $p < 0.05$, ** $p < 0.01$ compared with miR-155 EV or miR-155 inhibitor EV group in the same treatment method of each cell line.

infected cells with or without isoflurane treatment after LPS stimulation (Figure 5). These results suggested that overexpression of miR-155 impaired the effect of isoflurane and inhibition of miR-155 promoted the effect of isoflurane on LPS-induced cell injury.

4.5. HO-1 is involved in the effect of isoflurane *in vitro*

To investigate whether the protective effect of isoflurane on LPS-induced cell injury was related to a gene downstream of HIF1-ALPHA, we measured HO-1 protein by western blot. Postconditioning with isoflurane increased HO-1 protein levels compared to the LPS-treated group (Figure 2C). The change trend of HO-1 protein levels were the same as HIF1-ALPHA in MH-S and MLE-12 cells with different

treatment (Figure 5C, 5D). In addition, HO-1 protein decreased when HIF1-ALPHA was downregulated by siRNA (Figure 3D). We found that HIF1-ALPHA and HO-1 involved in the protective effect of isoflurane on cells treated with LPS. Overexpression of miR-155 alleviated the effect on HO-1 protein in LPS-treated cells. Downregulation of miR-155 increased HO-1 expression.

4.6. Postconditioning with isoflurane attenuates LPS-induced ALI

Twenty four hours after the administration of LPS by intraperitoneal injection, lung tissues of mice were harvested for observations of histopathological changes. The photomicrographs showed interstitial edema, intra-alveolar exudation,

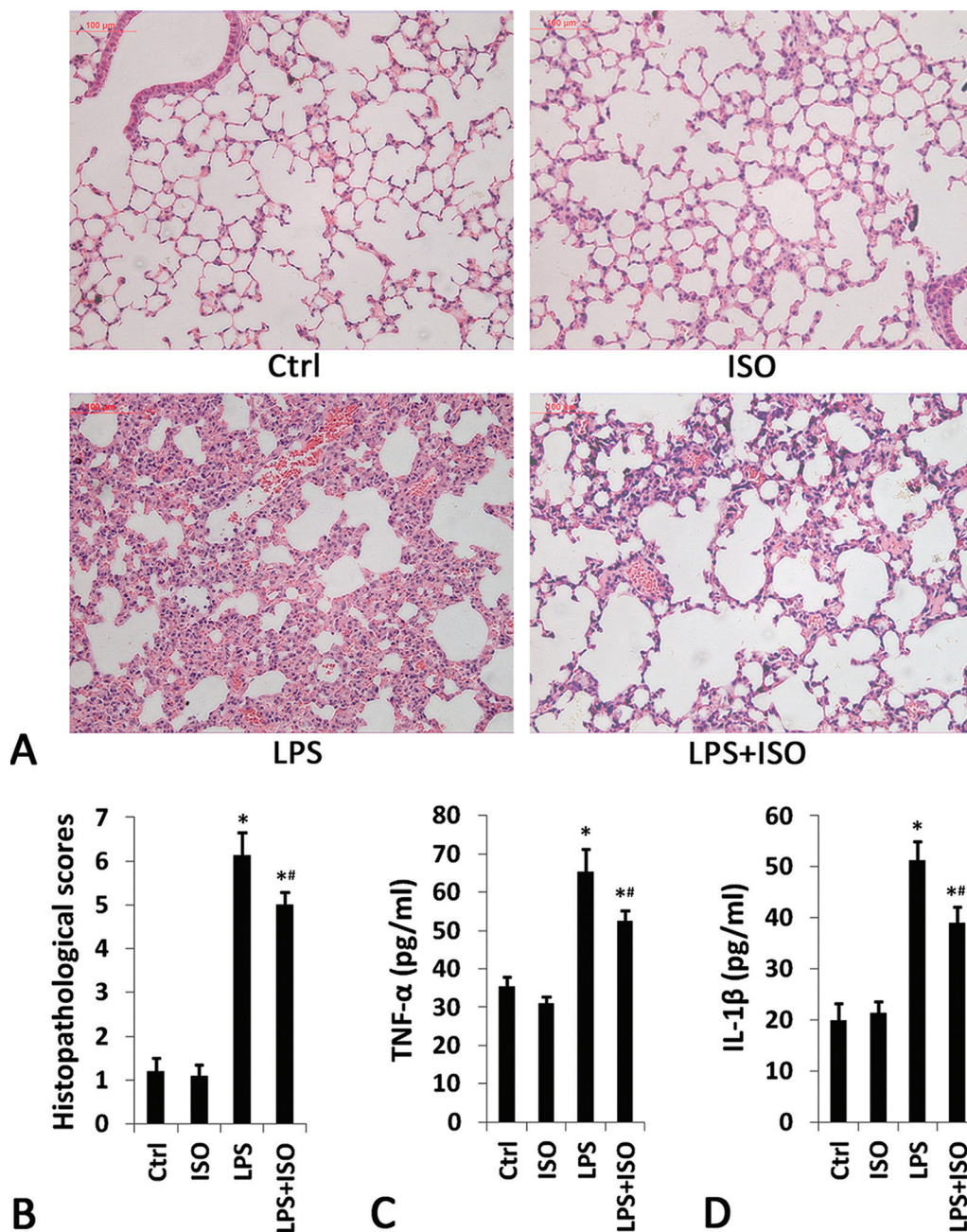


Figure 6. Effects of isoflurane on lung histology. Mice were injected with saline and postconditioning with 1.4% isoflurane for 4 h (ISO) or posttreated with 1.4% isoflurane for 4h after LPS stimulation for 24 h (LPS+ISO). The mice injected with saline and inhaled air were used as normal control (Ctrl), and mice posttreated with air for 4 h after receiving LPS injection for 24 h were used injury control (LPS). (A) Representative photomicrographs of lung tissues stained with hematoxylin and eosin (200×). (B) Histopathological scores were analyzed by lung injury score. (C-D) The levels of TNF-α (C) and IL-1β (D) in bronchoalveolar fluid (BALF) were detected by ELISA. * $p < 0.05$ compared with the Ctrl group, # $p < 0.05$ compared with the LPS group.

hemorrhage, alveolar wall thickening, and infiltration of inflammatory cells into the lung interstitium and alveolar space (Figure 6A). Postconditioning

with isoflurane ameliorated these effects. Semiquantitative evaluation using a lung injury score (32) showed that the degree of lung injury

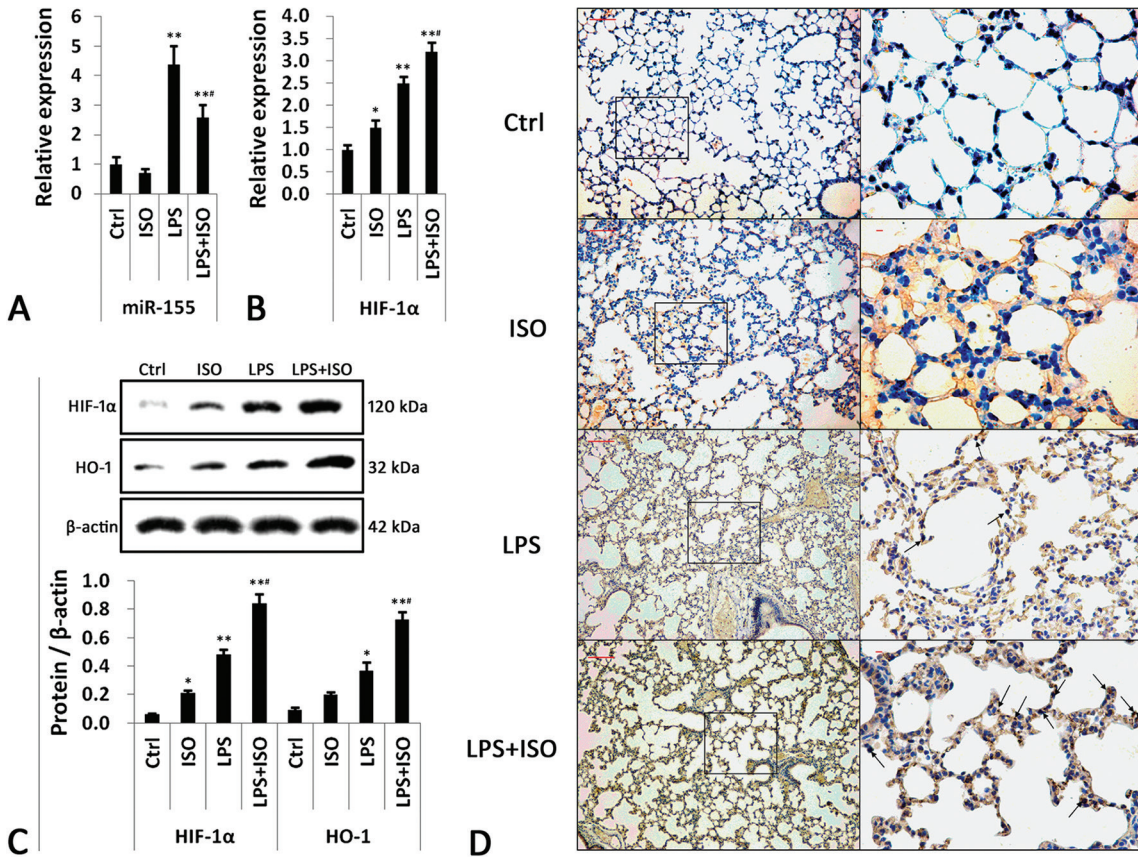


Figure 7. Effects of isoflurane on HIF-1α, HO-1, and miR-155 in the lung. Mice were treated as described above. (A-B) The levels of miR-155 (A) and HIF-1α mRNA (B) in lung tissues were measured by Q RT-PCR with U6 or level β-actin as an internal control. (C) The HIF-1α and HO-1 protein levels in lung tissues were assessed by western blotting, normalized to the levels of β-actin. **p* < 0.05, ***p* < 0.01 compared with the Ctrl group, ****p* < 0.05 compared with the LPS group. (D) Representative photomicrographs of lung tissues with immunohistochemical staining for HIF-1α (100× in left, 400× in right and black arrows show HIF-1α expression).

in the LPS-Isoflurane group was lower than that in the LPS group (Figure 6B). To determine isoflurane effects, we measured TNF-α and IL-1β levels in BALF. Isoflurane reduced the levels of TNF-α and IL-1β, suggesting that isoflurane was essential for attenuating LPS-induced ALI (Figure 6C, 6D).

4.7. Inverse expression of HIF1-ALPHA and miR-155 after postconditioning with isoflurane

Levels of HIF1-ALPHA mRNA and miR-155 were measured by Q RT-PCR (Figure 7A, 7B). HIF1-ALPHA protein in lung tissue after LPS induction of ALI was determined by western blot and immunohistochemistry after 4 h isoflurane treatment (Figure 7C, 7D). HO-1 protein was also measured by western blot (Figure 7C). The level of miR-155 increased significantly in LPS

injection group compared with the normal control group, while decreased significantly in the group posttreated with isoflurane after challenge with LPS compared with the LPS group (Figure 7A). And postconditioning with isoflurane increased HIF1-ALPHA mRNA and protein levels after LPS treatment (Figure 7B, 7C, 7D).

4.8. Overexpression of miR-155 impaired isoflurane protection against LPS-induced injury

To explore whether isoflurane exerted a protective effect by suppressing miR-155 activity, TNF-α and IL-1β levels were measured in BALF of mice that were overexpressing or downregulating miR-155. The levels of TNF-α and IL-1β were higher in the miR-155-overexpressing group compared with mice of control group. In addition, TNF-α and IL-1β

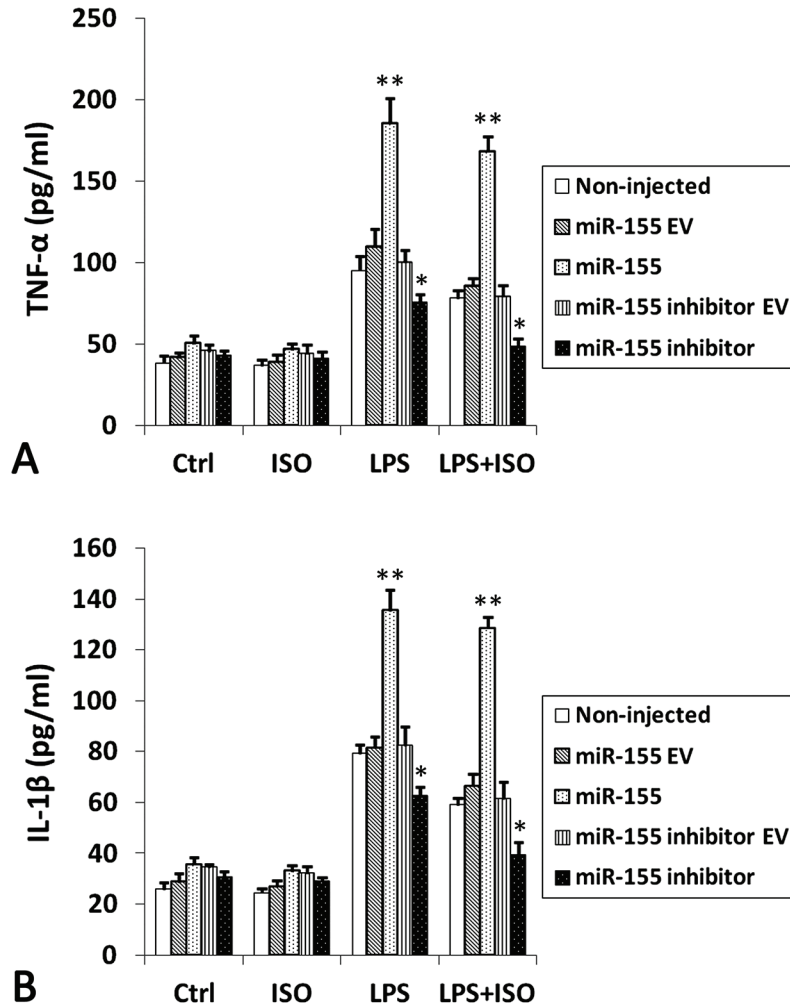


Figure 8. Effect of upregulating or downregulating miR-155 on protection by isoflurane against LPS-induced ALI *in vivo*. Mice were injected via tail veins with 2.5×10^7 TU miR-155, miR-155 inhibitor, miR-155-EV, or miR-155 inhibitor-EV lentiviruses respectively. Fourteen days after injection, mice in each group were divided into four groups and treated as described above. The levels of TNF- α (A) and IL-1 β (B) in bronchoalveolar fluid (BALF) were detected by ELISA. Mice without injection of lentiviruses (Non-injected) in each treatment group were used as controls. * $p < 0.05$, ** $p < 0.01$ compared with Non-injected group in the same treatment method of mice

levels in BALF were lower in mice in which miR-155 was inhibited compared with mice in the control group of non-injected lentiviruses (Figure 8A, 8B). The results indicated that overexpression of miR-155 impaired the isoflurane protection against LPS-induced injury, while inhibition of miR-155 reduced the inflammatory reaction and promoted the isoflurane protection against LPS-induced injury.

5. DISCUSSION

In this study, we demonstrated that isoflurane induced cell proliferation and postconditioning with

moderate isoflurane attenuated LPS-induced cell injury and inflammatory in ALI after LPS challenge. The attenuation was due to upregulation of HIF-1 α through suppression of miR-155. Knocking down HIF-1 α with siRNA abrogated the protective effect that isoflurane had on LPS-induced cell injury. We showed that miR-155 was sensitive to isoflurane in LPS-treated cells and reduced significantly compared with the LPS-treated cells without isoflurane treatment, thereby releasing mRNA targets from its inhibitory effect. We also showed that miR-155 directly targeted HIF-1 α , and downregulation of miR-155 induced the upregulation of HIF-1 α during normoxia.

Overexpression of miR-155 prevented the positive effects of isoflurane on the LPS-induced inflammatory reaction. However, when we raised the expression of miR-155 during normoxia, we did not find complete inhibition of HIF-1 α , which would be supposed that HIF-1 α might be also regulated by other miRNAs relating to isoflurane effects (33). In both cells (MH-S and MLE-12) and mice treated with isoflurane, HO-1, a gene downstream of HIF-1 α , increased expression according to western blot results. These results indicated that isoflurane exerted protective effects through a pathway involving miR-155, HIF-1 α , and HO-1.

Isoflurane protects against LPS-induced dysfunction in crucial organs including the lung and brain (34-37). Isoflurane postconditioning reduces the release of systemic cytokines such as TNF- α and IL-1 β in rats (21). The anti-inflammatory effect of isoflurane was associated with upregulation of HIF-1 α . HIF-1 α is a critical transcription factor in modulating innate immunity in the lung (38, 39). The present study showed that after dealing with LPS, isoflurane, as a nonhypoxic mediator, induced HIF-1 α protein expression. When cell or lung stimulated with LPS, reactive oxygen species (ROS) were generated for eradicating bacteria and other pathogens (40). It was demonstrated that ROS upregulated the induction of HIF-1 α gene transcription involving in phosphatidylinositol 3-kinase-Akt/Protein kinase C/Histone deacetylase pathway (41). Hence, the induction of HIF-1 α might partially dependent on ROS for immune reaction when challenged with LPS. HIF-1 α is reported to exert a cytoprotective effect by regulating downstream genes (42). In a HIF-1 α -deficient model, macrophages showed dysfunctional bacterial killing and sustained inflammation (43). We previously reported that isoflurane posttreatment improved cecal ligation and puncture (CLP)-induced ALI by increasing expression of HO-1, a gene downstream of HIF-1 α (20). HO-1 mediates the protective effect of isoflurane and blocks the release of TNF- α in LPS-stimulated macrophages (22). In this study, we investigated the mechanism of these findings. We found a novel regulatory mechanism in which isoflurane controlled LPS-induced inflammation by downregulating miR-155, and further inducing the expression of HIF-1 α , resulting in elevation of the anti-inflammatory protein HO-1.

MiRNAs are crucial regulators of gene expression that are incorporated into RNA-induced silencing complexes (15). In our study, HIF-1 α was

proved as a target gene of miR-155. Transfection with miR-155 or miR-155 inhibitors significantly affected HIF-1 α mRNA and protein levels in MH-S and MLE-12 cells, indicating that miR-155 regulated HIF-1 α posttranscriptional expression. The importance of miR-155 in isoflurane regulation of inflammation was confirmed by upregulating or knocking down miR-155 expression. After treating with isoflurane, mice injected with miR-155 inhibitors ameliorated LPS-induced lung injury and secreted less TNF- α and IL-1 β compared with control mice. Overexpressing miR-155 blocked the protective effect of isoflurane, resulting in higher levels of cytokines in BALF.

HIF-1 α regulates downstream genes such as HO-1 by binding to hypoxia responsive elements. Our functional studies revealed that HO-1 expression was upregulated by LPS stimulation. As an isozyme of the rate-limiting enzyme in heme oxidative degradation, HO-1 has anti-inflammatory effects in LPS-induced ALI (44, 45). Overexpression of HO-1 inhibits pro-inflammatory cytokines in LPS-induced RAW264.7 cells (22). In our study, we found that HO-1 protein decreased significantly in cells treated with siRNA HIF-1 α when stimulating with LPS with or without isoflurane exposure. Overexpressing miR-155 reduced induction of HO-1 by isoflurane, consistent with results with HIF-1 α . These data illustrated that isoflurane postconditioning attenuated LPS-induced ALI and cell injury by targeting signaling through miR-155, HIF-1 α , and HO-1.

A potential limitation of our study was that respiratory physiological characteristics of mice might be altered by isoflurane conditioning. However, our previous study showed that preconditioning with 1 MAC of inhalation agent alone did not affect the acid-base status or parameters of arterial blood gases (23).

As a pivotal regulator of inflammation, miR-155 has numerous target genes involved in immune function such as SOCS1 and SHIP-1, two crucial anti-inflammatory regulators (46, 47). Previous studies demonstrated that miR-155 attenuates the TLR signal by targeting SOCS1 and blocks the TLR4 signal by reducing SHIP-1 (48, 49). Thus, isoflurane might also have anti-inflammatory effects by regulating SOCS1 or SHIP-1. Further investigations should be performed to explore potential mechanisms.

In summary, our investigation demonstrated that isoflurane posttreatment at 1 MAC for 4 hours

alleviated LPS-induced ALI and cell injury. The protective effect might be mediated by increasing cell proliferation and upregulating HIF-1 α expression through suppressing miR-155. HO-1, a gene downstream of HIF-1 α , seemed to be involved in the relevant signaling pathway. Further studies will be performed to explore whether other miRNAs participate in isoflurane regulation of ALI.

6. ACKNOWLEDGEMENTS

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- Abbreviations:** ALI, acute lung injury; LPS, lipopolysaccharide; HIF-1 α , Hypoxia inducible factor 1 alpha; HO-1, heme oxygenase-1; miRNAs, microRNAs; UTRs, untranslated regions; TLR, toll like receptor; H&E, hematoxylin and eosin; BALF, bronchoalveolar lavage fluid; CLP, cecal ligation and puncture
- Key Words:** Isoflurane, Posttreatment, Endotoxin, Acute Lung Injury, miR-155, HIF1 alpha
- Send correspondence to:** Hong Jiang. Department of Anesthesiology, Shanghai Ninth People's Hospital Affiliated to Shanghai Jiao Tong University, School of Medicine, No. 639 Zhi Zao Ju Road, Shanghai, China, Tel: 86-21-23271699, Fax: 86-21-23271699, E-mail: dr_jianghong@163.com