 Isoflurane suppresses lung ischemia-reperfusion injury by inactivating NF-κB and inhibiting cell apoptosis

NING LV¹ and XIAOYUN LI²

¹Department of Anesthesiology, Tianjin Central Hospital of Gynecology Obstetrics, Tianjin 300100; ²Department of Anesthesiology, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong 510630, P.R. China

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Abstract. Patients with lung ischemia-reperfusion injury (LIRI), involving cytokines, including interleukin (IL)-6 and IL-8, display poor clinical outcomes. Isoflurane displays protective effects against ischemia-reperfusion injury in numerous organs. In the present study, the effects of isoflurane on LIRI were investigated in vitro using a hypoxia-reoxygenation (HR) cell model. The mRNA expression levels of specific genes were analyzed by reverse transcription-quantitative PCR and protein expression levels were measured by ELISA and western blotting. Cell apoptosis and proliferation were assessed by flow cytometry and the Cell Counting Kit-8 assay, respectively. Isoflurane pretreatment decreased HR-induced IL-6 and IL-8 expression levels in A549 cells. Isoflurane pretreatment also inhibited HR-induced cell apoptosis and Bax expression, and reversed HR-induced downregulation of Bcl-2 expression. Moreover, isoflurane pretreatment decreased HR-induced NF-κB phosphorylated-p65 protein expression and NF-κB activation. Furthermore, HR-induced increases in malondialdehyde concentration and decreases in superoxide dismutase activity were reversed by isoflurane pretreatment. In conclusion, the results indicated that isoflurane suppressed LIRI by inhibiting the activation of NF-κB and the induction of cell apoptosis.

Introduction

Lung ischemia-reperfusion injury (LIRI) is an intricate pathological process that occurs during numerous clinical conditions, including lung transplantation, pulmonary embolism, resuscitation for circulatory arrest and cardiopulmonary bypass cardiac surgery (1,2). Lung transplantation, which induces the most severe form of LIRI, causes primary graft failure, leading to short- and long-term morbidity and mortality (3); therefore, novel therapeutic strategies are required to improve the clinical outcomes of patients with LIRI.

Ischemia-reperfusion (IR) induces inflammation and injury by rapidly activating the innate immune system (4). The mechanisms underlying LIRI involve the release of inflammatory cytokines and an increase in their expression levels, which results in cell damage, necrosis and apoptosis in the lungs (5,6).

In 1988, Warltier et al (7) reported that pretreatment of cells with isoflurane improved left ventricular systolic function following occlusion of the left anterior descending coronary artery for 15 min. To date, the protective functions of isoflurane against IR injury (IRI) have been confirmed by numerous studies. For example, Kehl et al (8) reported that low concentration isoflurane was sufficient to precondition myocardial tissue against infarction. In addition, Lv et al (9) indicated that pretreatment of rats with isoflurane ameliorated IR combined with lipopolysaccharide (LPS)-induced liver injury. Liang et al (10) reported that isoflurane pretreatment of rats also attenuated renal IRI by reducing inflammation and apoptosis. Furthermore, it has been reported that emulsified isoflurane pretreatment of rats ameliorated hepatic IR-induced lung injury (11). However, whether isoflurane attenuates LIRI via an anti-inflammatory mechanism and the inhibition of apoptosis is not completely understood; therefore, the present study aimed to investigate the mechanisms underlying the effects of isoflurane during LIRI.

Materials and methods

Cell culture and hypoxia-reoxygenation (HR) model. A549 cells are derived from a human alveolar cell carcinoma and are the most widely used in vitro model of type 2 pulmonary alveolar epithelial cells, possessing multiple properties of these cells (12). A549 cells (American Type Culture Collection) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 4.5 g/l glucose (Gibco; Thermo Fisher Scientific, Inc.), 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator with 5% CO₂ at 37°C.
A549 cells were pretreated with isoflurane (1.4% v/v) prior to HR induction according to a previously method (13). Briefly, cells were placed in a sealed acrylic chamber with a circular opening in which a rubber cannula was inserted and a mixture of 95% air and 5% CO₂ was delivered. Subsequently, isoflurane was delivered into the chamber using a vaporizer for 60 min. Untreated cells served as a control group. Capnography was conducted using the Networked Multiparameter Veterinary Monitor LifeWindow 6000V (Digicare Animal Health).

An *in vitro* model of LIRI was established by HR induction. A549 cells were placed in a hypoxic chamber (0% O₂) and incubated with 95% N₂ and 5% CO₂ for 25 min at room temperature. Subsequently, cells in the hypoxic chamber (95% N₂ and 5% CO₂) were incubated in a 37°C incubator for 3 h to establish hypoxia. A549 cells were transferred to a normoxic incubator with 5% CO₂ at 37°C for 1 h to induce reoxygenation. Following incubation under hypoxic conditions for 3 h, the partial percentage of O₂ in the culture media was 5% compared with 21% in the normoxic culture media.

**Cell proliferation assay.** Cells (3x10⁴ cells/well) were seeded into 96-well plates and cultured at 37°C with 5% CO₂. Cell proliferation was determined using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.).

**Cell apoptosis assay.** Cells (1x10⁶ cells/well) were seeded into 6-well plates and cultured at 37°C with 5% CO₂. Cells were stained at room temperature for 10 min in the dark using the Annexin V-FITC/PI Apoptosis Detection kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol.

**Detection of interleukin (IL)-8, IL-6, superoxide dismutase (SOD) and malondialdehyde (MDA) content.** IL-8 (cat. no. S80000C) and IL-6 (cat. no. S6050) ELISA kits (R&D Systems, Inc.) were used to measure IL-8 and IL-6 protein concentrations, according to the manufacturer's protocols. The induction of oxidative stress was evaluated using SOD (cat. no. A001-3) and MDA (cat. no. A003-1) assay kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol.

**Detection of NF-κB activity.** A549 cells (1x10⁶) were seeded into 24-well plates and transfected with the pBIIx-luc-dependent luciferase reporter construct (0.4 mg; GenScript Biotech Corporation) and the Renilla luciferase vector (Promega Corporation) using Lipofectamine™ 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following incubation for 24 h at 37°C, cells were pretreated with isoflurane and HR was induced according to the aforementioned protocol. Subsequently, luciferase activities were detected using a Dual-Luciferase Reporter assay system (Promega Corporation) according to the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity.

**Western blotting.** Western blotting was performed to measure the protein expression levels of NF-κB phosphorylated (p)-p65, NF-κB p65, Bax, Bcl-2 and proliferating cell nuclear antigen (PCNA). Total protein was extracted from A549 cells using cold RIPA buffer (Roche Diagnostics) and total protein was quantified using the Bicinchoninic Acid Protein Assay kit (Applygen Technologies, Inc.). Proteins (15 µg/lane) were separated via SDS-PAGE on 10% gels and transferred onto nitrocellulose membranes. The membranes were incubated overnight at 4°C with primary antibodies which were all purchased from Cell Signaling Technology, Inc. targeted against: Bax (cat. no. 2772; 1:1,000) and Bcl-2 (cat. no. 3498; 1:1,000), PCNA (cat. no. 13110; 1:1,000), NF-κB p-p65 (cat. no. 3033; 1:1,000), NF-κB p65 (cat. no. 8242; 1:1,000) and GAPDH (cat. no. 5174; 1:1,000). Following primary antibody incubation, the membranes were blocked in 5% non-fat milk at room temperature for 1 h and incubated with anti-rabbit IgG secondary antibody (cat. no. 7074; 1:10,000; Cell Signaling Technology, Inc.) at room temperature for 2 h. Protein bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific, Inc.) using the Odyssey Infrared Imaging system (LI-COR Biosciences). Densitometry was quantified by ImageJ software (version. 1.8.0; National institutes of Health). GAPDH was used as the loading control.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from A549 cells using the Easystep Universal RNA Extraction kit (Promega Corporation) and RNA concentration was quantified using a NanoDrop spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA at 37°C for 10 min followed by incubation at 85°C for 5 sec using the M-MLV reverse transcriptase [50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM MgCl₂, 1 mM DTT, 0.5 mM [H]dTTP, 0.1 mM poly(A), 0.1 mM oligo(dT)₁₂₋₁₈, 0.1 mg/ml BSA and reverse transcriptase, Thermo Fisher Scientific, Inc.]. Subsequently, qPCR was performed using SYBRGreen Master Mix (Promega Corporation) and the Real-Time PCR system (Bio-Rad Laboratories, Inc.). The primer sequences were as listed: PCNA forward, 5'-CGGTGAAACCTCACA GTATGT-3' and reverse, 5'-TCTTCGCCCCTTAGTGA ATGAT-3'; BAX forward, 5'-CACACGCTCTGAAACAGAT CATGA-3' and reverse, 5'-TACGCCCCATCTTCTTCCA GTATG-3'; Bcl-2 forward, 5'-CACCCTGTCATCCTTCTC TT-3' and reverse, 5'-AGCGTCTTCCAGACAGCCAG-3'; IL-6 forward, 5'-AGCCACCTACCTCTTTCAAGCAAGA-3' and reverse, 5'-TACTCTCTGCGACAGCTTGGCCT-3'; IL-8 forward, 5'-ATGACTTTCCAAGCTGGCCTGTTG-3' and reverse, 5'-TCTCAGCCCCCTTTCAAATACCTTCT-3'; RELA forward, 5'-CCACAGAGGTTGGAGAACAGG-3' and reverse, 5'-GGATCCCCAGGTTCTGGAAAAC-3'; IkBa forward, 5'-ACCCTGTTGTCTACCTTGTTA-3' and reverse, 5'-CTGCTGTCTGTATCCGGGTG-3'; IkBα forward, 5'-GAT ATCGCCCTGATCTGGCT-3' and reverse, 5'-AGGTGTTGC CCTGACATCAC-5'; and GAPDH forward, 5'-TGTGTATCG TGGAAGGAC-3' and reverse, 5'-TGTCATCATATTTGG CAGGT-3'. The thermocycling conditions were as listed: 94°C for 30 sec; followed by 40 cycles of 94°C for 5 sec, 60°C for 15 sec and 72°C for 10 sec. mRNA expression levels were
Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS software (version 14.0; SPSS, Inc.). Comparisons among groups were analyzed by one-way ANOVA followed by Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Isoflurane pretreatment reverses HR-induced reductions in cell proliferation. The effects of isoflurane on cell proliferation

Figure 1. Isoflurane pretreatment reverses HR-induced reductions in cell proliferation. (A) Cell proliferation was assessed. (B) mRNA and (C) protein expression levels of PCNA were detected. (D) PCNA protein expression was semi-quantified. *P<0.05 and **P<0.01 vs. Ctrl; †P<0.05 vs. HR. Ctrl, control; HR, hypoxia-reoxygenation; PCNA, proliferating cell nuclear antigen.

Figure 2. Isoflurane pretreatment inhibits HR-induced cell apoptosis. The rate of apoptosis was (A) determined by flow cytometry and (B) quantified. ‡P<0.01 vs. Ctrl; §P<0.05 vs. HR. Ctrl, control; HR, hypoxia-reperfusion; PI, propidium iodide.

quantified using the 2−ΔΔCq method (14) and normalized to the internal reference gene GAPDH.
were assessed. Cell proliferation in the HR group was significantly decreased compared with the control group, whereas pretreatment with isoflurane reversed the effects of HR on cell proliferation (Fig. 1A).

In addition, the expression levels of the cell proliferation-associated molecule, PCNA, were assessed in the different groups by RT-qPCR and western blotting. The mRNA expression levels of PCNA were significantly decreased in the HR group compared with the control group (Fig. 1B). Conversely, pretreatment with isoflurane increased PCNA mRNA expression in the HR group (Fig. 1B). PCNA protein levels displayed a similar pattern to PCNA mRNA levels (Fig. 1C and D).

Isoflurane pretreatment inhibits HR-induced cell apoptosis. Subsequently, the effects of isoflurane on cell apoptosis were investigated. The rate of apoptosis was significantly increased in the HR group compared with the control group, and pretreatment with isoflurane significantly reversed HR-induced cell apoptosis (Fig. 2).

Isoflurane pretreatment reverses HR-induced increases in the Bax/Bcl-2 ratio. Furthermore, the expression levels of apoptosis-related proteins Bax and Bcl-2 were examined in the different groups by RT-qPCR and western blotting. The mRNA expression levels of Bax were significantly higher in the HR group compared with the control group, whereas the opposite effect was observed for Bcl-2. Pretreatment with isoflurane significantly reversed the HR-induced effects on apoptosis-related genes (Fig. 3A and B). The western blotting results indicated that the protein expression levels of Bax and Bcl-2 displayed a similar trend to the mRNA expression levels (Fig. 3C and D).

Isoflurane pretreatment reverses HR-induced increases in MDA concentration and decreases in SOD activity.

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Figure 3. Isoflurane pretreatment reverses HR-induced increases in the Bax/Bcl-2 ratio. mRNA expression levels of (A) Bax and (B) Bcl-2. Bax and Bcl-2 protein expression levels were (C) determined by western blotting and (D) semi-quantified. *P<0.01 vs. Ctrl; †P<0.05 vs. HR. Ctrl, control; HR, hypoxia-reoxygenation.

Figure 4. Isoflurane pretreatment reverses HR-induced increases in MDA concentration and decreases in SOD activity. (A) MDA concentration and (B) SOD activity were detected. **P<0.01 vs. Ctrl; †P<0.05 vs. HR. Ctrl, control; HR, hypoxia-reoxygenation; MDA, malondialdehyde; SOD, superoxide dismutase.
Subsequently, the effects of isoflurane on reactive oxygen species (ROS)-associated markers, including SOD activity and MDA levels, were investigated. Significantly higher MDA concentrations and lower SOD activity levels were observed in the HR group compared with the control group; however, pretreatment with isoflurane significantly reversed these effects (Fig. 4A and B).

**Isoflurane pretreatment reduces HR-induced inflammatory cytokine release.** The effect of isoflurane on the production of inflammatory cytokines (IL-6 and IL-8) was assessed. Pretreatment with isoflurane significantly reduced the release of IL-6 and IL-8 compared to the HR group (Fig. 5). The expression levels of IL-6 and IL-8 were measured using qPCR and ELISA. Pretreatment with isoflurane significantly reduced the expression and production of IL-6 and IL-8 (Fig. 5A and B).

**Isoflurane pretreatment suppresses HR-induced NF-κB activation.** The activity of NF-κB, a key regulator of inflammatory responses, was investigated. Pretreatment with isoflurane significantly suppressed the expression and activity of NF-κB (p65) in the HR group (Fig. 6). The expression levels of the RELA and IκBα genes were measured using qPCR, and the protein levels of p65 were determined by western blotting and semi-quantified (Fig. 6A-C and D). NF-κB activity was assessed using a dual-luciferase reporter assay (Fig. 6E and F). Pretreatment with isoflurane significantly reduced the expression and activity of NF-κB (p65) compared to the HR group.
and release of inflammatory cytokines, including IL-8 and IL-6, was examined in A549 cells and the cell culture medium, respectively. The mRNA expression levels and protein concentrations of IL-8 and IL-6 in the HR group were significantly increased compared with the control group; however, pretreatment with isoflurane significantly reduced the expression and production of IL-8 and IL-6 compared with the HR group (Fig. 5).

**Isoflurane pretreatment suppresses HR-induced NF-kB activation.** To investigate the effect of isoflurane on the expression of NF-kB-associated genes, RT-qPCR was performed. Increased levels of NF-kB inhibitor α (IkBa) and IkBI were observed in the HR group compared with the control group. Pretreatment with isoflurane upregulated the mRNA expression levels of IkBa and IkBI, whereas these effects were not observed for RELA proto-oncogene, NF-kB subunit (RELA; Fig. 6A-C).

NF-kB activity was determined following HR and isoflurane pretreatment by western blotting and the dual-luciferase reporter assay. The HR group displayed significantly increased p-p65 expression levels compared with the control group, which were significantly decreased by isoflurane pretreatment (Fig. 6D and E). Furthermore, pretreatment with isoflurane significantly suppressed the enhanced NF-kB activation in the HR group (Fig. 6F).

**Discussion**

LIRI is the second most common cause of respiratory insufficiency (15,16). Although the current treatment strategies used for lung protection are effective, they may not be sufficient to prevent LIRI (17); therefore, identifying a novel strategy to protect against LIRI is required.

Apoptosis is the process of programmed cell death and is associated with the pathogenesis of LIRI. It has been reported that the inhibition of apoptosis may ameliorate LIRI (18). The present study suggested that LIRI induced an increased rate of apoptosis compared with the control group, which was significantly reversed by pretreatment with isoflurane. Furthermore, the expression levels of apoptosis-related markers, including Bcl-2 and Bax, were investigated. The Bcl-2 family can be divided into three subgroups that modulate cell apoptosis: Anti-apoptotic Bcl-2, proapoptotic Bax and the BH3-only subfamily (19,20). The results indicated that LIRI induced significantly higher Bax expression and reduced Bcl-2 expression compared with the control group, and pretreatment with isoflurane significantly reversed LIRI-induced effects.

In a previous study, isoflurane pretreatment reduced injury to normal lung cells in Sprague-Dawley rats by regulating tumor necrosis factor-α, intercellular adhesion molecular-1 and NF-kB (11). In the present study, isoflurane pretreatment reversed LIRI-induced reductions in cell proliferation. In addition, the expression of PCNA, a cell proliferation-associated marker (21), was significantly reduced in the LIRI group compared with the control group, which was reversed by isoflurane pretreatment.

During LIRI, the imbalance between the demand and supply of pulmonary oxygen leads to oxidative stress (2), which leads to an excessive accumulation of ROS (16,22). MDA is the final product of peroxidation, and SOD is an antioxidant enzyme that protects the epithelium/endothelium in the lung from oxidant injury and inflammation (23,24). The results of the present study demonstrated that LIRI increased MDA levels and decreased SOD activity, and that these effects were reversed by isoflurane pretreatment.

Moreover, the infiltrating ability of inflammatory cells in the lungs during LIRI is considered a crucial source of ROS production (16,25). LIRI is associated with the expression of IL-6 and IL-8 in small airway epithelial cells (26). Isoflurane decreased LPS-induced production of proinflammatory cytokines in rats, including IL-6 (27). A similar protective role was identified in a rat model of renal IRI (28). The present study indicated that the expression levels of IL-8 and IL-6 in the LIRI group were significantly higher compared with the control group, which was reversed by isoflurane pretreatment.

NF-kB is an important transcription factor that is involved in inflammation and LIRI; when activated, NF-kB promotes the expression of various inflammatory molecules, including cytokines, chemokines and adhesion molecules (29,30), contributing to lung injury. Sevoflurane pretreatment of the heart tissue decreased NF-kB activation and IR-induced production of inflammatory mediators, thus attenuating myocardial IRI (31). Emulsified isoflurane pretreatment of A549 cells displayed a similar effect on NF-kB activation. In the present study, higher mRNA expression levels of IkBa, IkBI and RELA (an NF-kB subunit) were observed in the LIRI group compared with the control group. Isoflurane pretreatment significantly increased the expression levels of IkBa and IkBI, whereas this effect was not observed for RELA expression. In addition, significantly increased levels of p-p65 NF-kB were observed in the LIRI group compared with the control group; however, isoflurane pretreatment decreased LIRI-induced effects on p-p65. Furthermore, the dual-luciferase reporter assay suggested that isoflurane pretreatment inactivated NF-kB hyperactivation in the LIRI group.

Collectively, the results indicated that isoflurane suppressed LIRI by inhibiting the activation of NF-kB and the induction of cell apoptosis, suggesting that isoflurane may serve as a therapeutic agent for LIRI.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

NL and XL carried out the experiments and analyzed the data. NL prepared the manuscript. All authors read and approved the final manuscript for publication.
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