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ORIGINAL ARTICLE

The preconditioning pulmonary protective effect of volatile isoflurane in acute lung injury is mediated by activation of endogenous iNOS

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Abstract
Purpose There is still a lack of evidence to support the use of specific anesthetic agents during major operations that could affect the development of postoperative acute lung injury (ALI). This study determined the protective effect of inhaled isoflurane in a rat model of endotoxin-induced ALI.
Methods Rats were exposed to volatile isoflurane (1.5 % in oxygen) or pure oxygen via a facemask for 2 h. After a 3-h recovery period, rats were reanesthetized and ALI was induced by intratracheal instillation of lipopolysaccharide (LPS, 1 mg/kg in 0.5 ml saline). In some animals, a specific inducible nitric oxide synthase (iNOS) inhibitor, 1400W, (10 mg/kg, i.p.) was administered before exposure to isoflurane. Animals were sacrificed 12 h later for analysis. Pulmonary artery vasomotor function and alveolo-capillary permeability were assessed. Expression of iNOS and CD11b, and activity of myeloperoxidase in the lung were analyzed.
Results The maximal relaxation response to acetylcholine was significantly potentiated in rats pretreated with isoflurane. Lung wet-to-dry ratio was reduced in the lung of isoflurane-treated animals. Expression of iNOS and CD11b were attenuated in the lung tissue obtained from rats receiving isoflurane. Furthermore, enzymatic activity of myeloperoxidase was also reduced in the lung preexposed to isoflurane. However, these pulmonary protective effects of isoflurane were significantly abolished by pretreatment with 1400W.
Conclusion Pretreatment with volatile isoflurane attenuated inflammatory process in the lung tissue of rats with LPS-induced ALI, and this preconditioning pulmonary protective effect was mainly mediated by activation of endogenous iNOS in the lung.

Keywords Inhaled anesthetics · Endotoxin · Myeloperoxidase · Inflammation

Introduction

Acute lung injury (ALI) and the subsequent acute respiratory distress syndrome (ARDS) are not uncommonly developed in the postanesthesia period, especially in patients with sepsis, multiple trauma/fracture, massive blood transfusion, burn, cardiopulmonary bypass, and inappropriate ventilatory setting [1]. The pathological manifestations of ALI/ARDS are characterized by diffuse endothelial injury, increased capillary permeability, and activation of inflammatory response [2]. The net changes on pulmonary vasculature and alveoli are increased intrapulmonary shunt fraction, decreased lung compliance, increased airway resistance, and ventilation/perfusion mismatch, resulting in a high mortality rate (30–40 %) [3]. The pulmonary protective effect of specific anesthetic techniques or agents during a major operation has been an important research issue in the prevention of postoperative ALI [4, 5]. Due to its minimal cardiac depression and coronary artery dilation effects [6], volatile isoflurane

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(2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) is one of the most commonly used general anesthetic agents during major surgeries, especially during cardiovascular procedures. Apart from maintaining anesthesia, isoflurane also mediates cytoprotective properties following ischemia–reperfusion injury and inflammatory response [7, 8]. Therefore, this study was performed to investigate the protective effect of inhaled isoflurane on pulmonary endothelial function and inflammatory response in a rat model of endotoxin-induced ALI.

Materials and methods

Administration of isoflurane and rat model of ALI

Sprague–Dawley rats (approximately 250 g) were obtained from the Animal Center of the National Cheng Kung University (Tainan, Taiwan) and were anesthetized by intraperitoneal injection of ketamine (35 mg/kg). A custom-built oronasal mask was connected to oxygen gas containing isoflurane (1.5 % v/v), and the animals were allowed to breath spontaneously through the oronasal mask for 2 h [9, 10]. We also determined the optimal concentration and exposure time for isoflurane in the cultured normal human bronchial epithelial cells (NHBEs). Concentration- and time-dependent responses (0–5 % isoflurane and 0–5 h exposure time to 1.5 % isoflurane, respectively) were tested in the NHBEs under a closed-circuit exposure chamber. Our in vitro studies demonstrated that exposure of cultured NHBEs to 1–2 % isoflurane for 2 h in oxygen causes significant upregulation of inducible nitric oxide synthase (iNOS) expression (Fig. 1). Therefore, the study tested the preconditioning protective effect of isoflurane at the concentration of 1.5 % for 2 h in the in vivo experiments. In the control group, animals received only pure oxygen via the mask. Rats were then allowed to recover from anesthesia. After a 3-h recovery period, lipopolysaccharide (LPS, *Escherichia coli* 055:B5; Sigma, St. Louis, MO, USA) solution (1 mg/kg in 300 μl distilled water) was intratracheally injected into the tracheobronchial tree of each animal to induce ALI under intraperitoneal anesthesia with ketamine [11]. Animals were sacrificed 12 h later by intraperitoneal injection of pentobarbital (250 mg/kg). Pulmonary artery and lung tissue were excised for laboratory analysis. In some animals, a specific iNOS inhibitor, 1400W, (10 mg/kg) was administered via intraperitoneal injection 30 min before inhalation of isoflurane to suppress the endogenous activity of iNOS [12]. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (The National Cheng Kung University, Tainan, Taiwan).

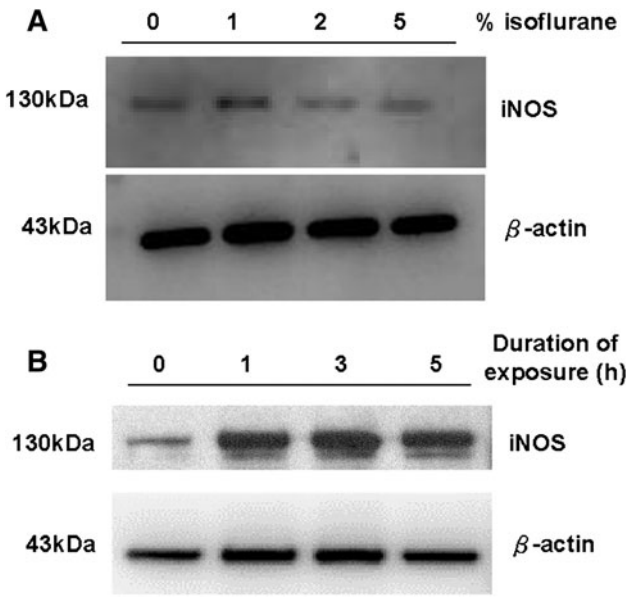

Fig. 1 Cultured epithelial cells [normal human bronchial epithelial cells (NHBEs)] were treated with different concentrations of isoflurane (% v/v) in serum-free cultured medium for 2 h (**a**) and were treated with 1.5 % (v/v) isoflurane in serum-free cultured medium for 0, 1, 3, and 5 h (**b**). The expression of endogenous inducible nitric oxide synthase (iNOS) was determined by Western blotting; representative blots are shown

Measurement of vascular reactivity

Second-generation pulmonary arteries were isolated and mounted in organ chambers containing 25 ml of Krebs solution [13]. The chambers were maintained at 37 °C and aerated continuously with 94 % oxygen (O_2)/6 % carbon dioxide (CO_2). Changes in isometric force were recorded continuously using an isometric force–displacement transducer (Grass FT03; Grass Instrument). Each ring was gradually stretched to 1.5 g. After a 45-min equilibration period, the rings were contracted by cumulative addition of phenylephedrine (10^{-9} to 10^{-5} M). To study the endothelial-dependent relaxation of the pulmonary artery, concentration–response curves were obtained by cumulative addition of acetylcholine (10^{-9} to 10^{-5} M) during contraction to half maximal effective concentration (EC_{50}) of phenylephedrine.

Lung wet-to-dry ratio (LWDR)

The right lung was excised and weighed immediately. Lung tissues were dried in an oven at 80 °C for 12 h and reweighed [14]. The LWDRs were obtained by dividing the mass of the initial specimen by the mass of the dried specimen.

Myeloperoxidase assay

Lung tissue was homogenized in potassium phosphate buffer solution and the resulting pellets were suspended in

50 mM hexadecyltrimethylammonium bromide. The samples were thawed and frozen four times before mixing with *O*-dianisidine dihydrochloride and hydrogen peroxide. The solution then proceeded to absorbance detection at 460 nm at regular intervals for 2 min.

Western blotting

Soluble protein extracts of lung homogenate (50 μg) were loaded into polyacrylamide gels and transferred onto nitrocellulose membranes. Anti-iNOS and anti-CD11b (an abundant complement receptor on neutrophils and monocytes) antibodies were used. After incubation with horseradish peroxidase (HRP)-linked secondary antibodies, bands were visualized using enhanced chemiluminescence.

Histological examination

Biopsies of formalin-fixed lung tissues were embedded in paraffin wax and sectioned (5 μm). Sectioned tissues were stained with hematoxylin and eosin (H&E) and observed under a light microscope.

Statistical analysis

Unless otherwise specified, results are presented as mean ± standard deviation (SD). Data were compared using the Mann–Whitney rank sum test, unpaired *t* test, or analysis of variance (ANOVA), as appropriate. Statistical significance was accepted at a level of $P < 0.05$.

Results

Contraction responses of isolated pulmonary artery were enhanced in rats treated with isoflurane (Fig. 2a), whereas the endothelium-dependent relaxation response to high concentrations of acetylcholine was significantly potentiated (Fig. 2b). Inhalation of isoflurane also significantly reduced lung water content and myeloperoxidase activity in lung tissue (Fig. 3a, b), but tissue hemoglobin levels were not different between treatment groups (Fig. 3c). Furthermore, protein expressions of iNOS and CD11b were attenuated in lung homogenates of rats pretreated with isoflurane (Fig. 4). In the absence of LPS challenge, exposure to volatile isoflurane enhanced the baseline expression of iNOS in the rat lung, but protein levels of iNOS were attenuated following LPS administration in these isoflurane-pretreated animals (Fig. 5). Due to these speculated findings, the pulmonary protective effect of isoflurane was examined in animals that received iNOS antagonist 1400W. Intraperitoneal injection of 1400W

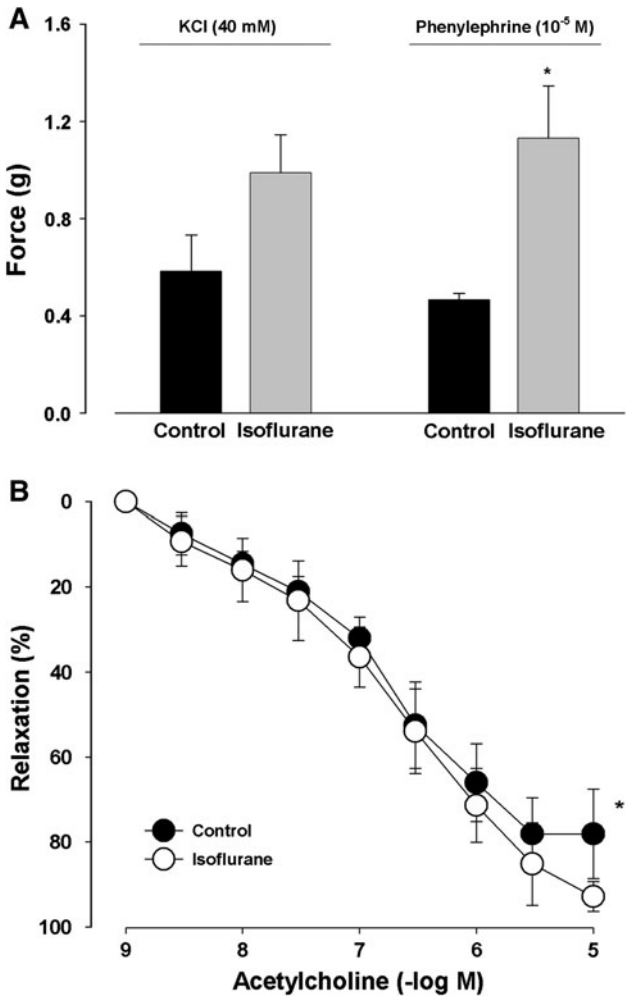

Fig. 2 Measurement of isometric force of isolated pulmonary artery segments. **a** The force of contraction responses to potassium chloride (KCl) (40 mM) and phenylephedrine (10^{-9} to 10^{-5} M) were enhanced in the isoflurane-treated group. **b** Endothelium-dependent relaxation induced by acetylcholine was also significantly potentiated in the pulmonary artery of isoflurane-treated animals. $^{*}P < 0.05$, $n = 5$–6 different groups. Data were analyzed by analysis of variance and are presented as mean ± standard deviation

suppressed the expression of endogenous iNOS in the lung of rats receiving inhaled isoflurane, indicating the systemic antagonism effect of the compound (Fig. 6). Administration of 1400W significantly abolished the preconditioning pulmonary protective effect of isoflurane by means of increased LWDR and myeloperoxidase activity (Fig. 3a, b). Under light microscopy, formation of hyaline membrane and infiltration of inflammatory cells were reduced in the lung tissue of rats preexposed to isoflurane (Fig. 7a–d). However, these histological improvements in the lung of isoflurane-treated animals were abrogated following systemic antagonism of iNOS (Fig. 7e–f).

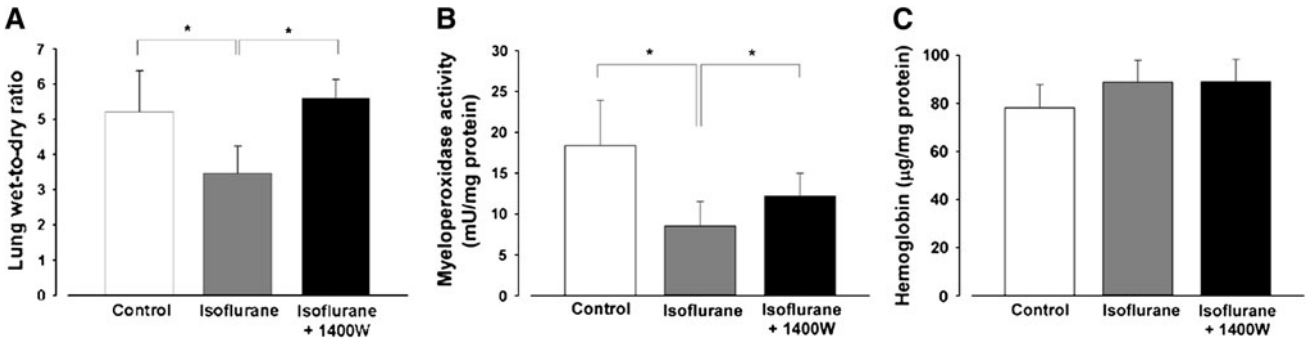

Fig. 3 **a** Degree of pulmonary edema was determined by measuring lung water content and is shown as lung wet-to-dry ratio (LWDR) in rats with acute lung injury. **b** Accumulation of neutrophils in the lung tissue of rats with acute lung injury (ALI) was determined by the activity of myeloperoxidase (MPO) as a quantitative measurement of lung inflammatory response. **c** Degree of lung hemorrhage was determined by tissue hemoglobin levels. These quantitative measurements showed that pretreatment with isoflurane significantly attenuated lung water content and MPO activity, but the degrees of pulmonary edema and inflammatory cell infiltration were enhanced in the lung tissue of animals receiving inducible nitric oxide synthase (iNOS) inhibitor 1400W, indicating that activation of endogenous iNOS is a major biological regulator for isoflurane-mediated preconditioning pulmonary protection effect. Degrees of lung hemorrhage were similar between the three treatment groups. $P < 0.05$ compared with isoflurane-only group; $n = 4$–5 different animals in each group. Data were analyzed by analysis of variance and are presented as mean ± standard deviation

Discussion

The pulmonary protective effect of inhaled anesthetics was first reported by Giraud et al. in a rat model of endotoxin-induced ALI. The authors showed that delivery of halothane (≥1 % v/v) significantly attenuated infiltration of polymorphonuclear neutrophils (PMNs) and reduced inflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-6, and macrophage inflammatory protein (MIP)-2) in bronchoalveolar lavage and lung homogenate [15]. This important nonanesthetic effect of inhalational halogenated gases was confirmed by Reutershan et al. [4], who demonstrated that pretreatment with isoflurane (1 or 12 h before an endotoxin challenge) attenuated recruitment of neutrophils into lung tissues and reduced degrees of pulmonary microvascular protein leakage following aerosolized LPS in mice [4]. Other investigators further affirmed that subanesthetic dose of isoflurane (0.7 % v/v) not only alleviated the degree of ALI induced by zymosan injury, but also significantly improved the overall survival rate of mice at day 7 after multiple organ injury [16]. In a murine model of septic peritonitis, Lee et al. [10] demonstrated that isoflurane anesthesia significantly improved renal and hepatic function at 24 h after injury, and the isoflurane-pretreated animals had significantly prolonged survival time than mice anesthetized with pentobarbital.

In our study, we used different approaches to investigate the antiinflammatory properties of inhalational isoflurane during preconditioning administration in a rat model of endotoxin-induced ALI. We demonstrated that preexposure to isoflurane significantly reduced the expression of iNOS and CD11b in the lung homogenates of rats with ALI. Activations of endogenous expression of iNOS and CD11b

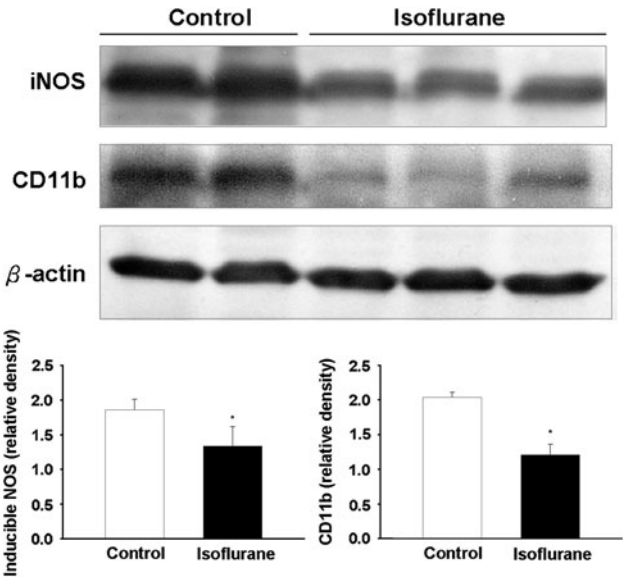

Fig. 4 Protein expression of inducible nitric oxide synthase (iNOS) and CD11b (a complement receptor on neutrophils) in the lung homogenate of rats with lipopolysaccharide-induced acute lung injury (ALI). Expressions of iNOS and CD11b were attenuated in lung tissue of rats pretreated with isoflurane, indicating that infiltration of polymorphonuclear neutrophils (PMNs) in lung parenchyma and inflammatory cascade were reduced. $P < 0.05$ compared with control group; $n = 4$–5 different animals in each group. Data were analyzed by unpaired t test and are presented as mean ± standard deviation

are sensitive biomarkers for the development of ALI in humans and in animal models [17–19]. Expression of the adhesion integrin CD11b in activated granulocytes mediates important proinflammatory reaction in lung injury [20]. Pharmacological inhibition of iNOS has been shown to attenuate systemic and regional lung inflammatory

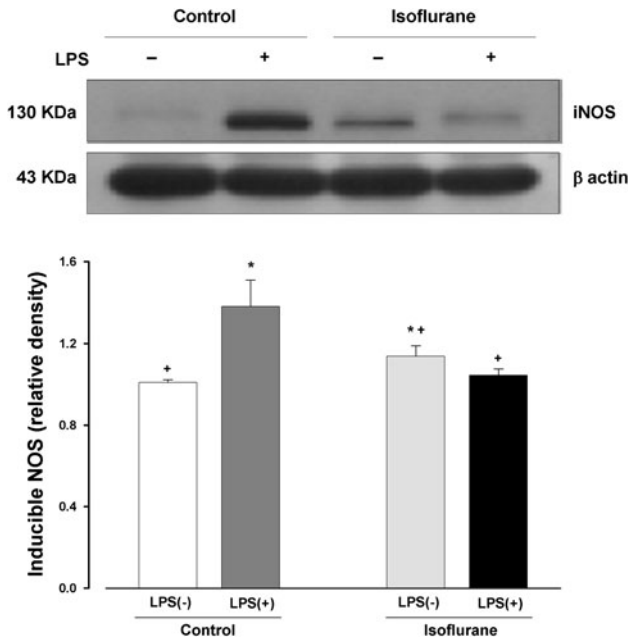

Fig. 5 Representative Western blots demonstrate protein expression of inducible nitric oxide synthase (iNOS) in the lung of rats receiving transtracheal injection of normal saline or lipopolysaccharide (LPS). Pretreatment with isoflurane upregulated the expression of iNOS in the absence of LPS challenge and inversely suppressed iNOS expression following LPS instillation. $^{*}P < 0.05$ compared with control animals receiving normal saline injection. $^{+}P < 0.05$ compared with control animals receiving LPS injection; $n = 3$–4 different animals in each group. Data were analyzed by analysis of variance and are presented as mean ± standard deviation

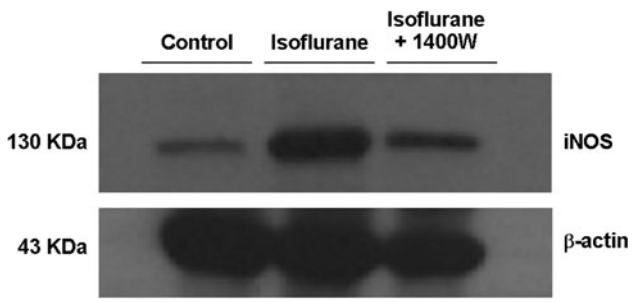

Fig. 6 Western blots demonstrate that protein expression of inducible nitric oxide synthase (iNOS) in the lung homogenate was upregulated by inhaled isoflurane (1.5 %), and intraperitoneal injection of 1400W significantly suppressed iNOS expression, indicating the presence of pharmacological inhibition of the activity of endogenous iNOS

responses following intravenous administration of LPS [21]. However, increased evidence underscores that enhancement of endogenous iNOS expression can be an important mechanistic pathway responsible for the organ protective effect of certain pharmacologic agents [22]. Apart from protein expression, we also demonstrated that pretreatment with isoflurane significantly suppressed the activity of myeloperoxidase in the lung of rats with ALI. Increased pulmonary iNOS expression is also associated with abnormal vasoreactivity in the isolated pulmonary artery. Generation of superoxide anions due to activation of iNOS and other proinflammatory genes might neutralize endothelium-derived NO, thereby enhancing contraction and impairing endothelium-dependent relaxation responses [13]. Furthermore, preconditioning with isoflurane abated the formation of hyaline membrane and infiltration of inflammatory cells in the lung of rats with endotoxin-induced ALI. The net effect of improved pulmonary endothelial function and lung inflammatory response in animals pretreated with isoflurane was preserved integrity of alveolocapillary barrier and resulted in reduced lung water content.

In the absence of LPS challenge, we detected that inhalation of isoflurane upregulated baseline expression of iNOS in the rat lung. Following intratracheal instillation of LPS, preexposure to isoflurane attenuated the protein expression of iNOS in the lung homogenate. We thus emphasized that the preconditioning pulmonary protective effect of isoflurane might be mediated via activation of endogenous iNOS. Therefore, a third experimental group was added to the study. Rats received intraperitoneal injection of a specific iNOS inhibitor 1400W before inhalational administration of isoflurane to antagonize the biological activity of endogenous iNOS. Antagonism of iNOS activity significantly diminished the pulmonary protective effect of isoflurane, as evidenced by increased myeloperoxidase activity, lung water content, and pathological injury in lung tissue. In fact, these findings are, in part, consistent with a previous study reported by Li et al. [23], who clearly demonstrate that isoflurane preconditioning enhanced pulmonary gas exchange function, ameliorated lung inflammation response, reduced lung edema/Evans blue dye extravasation, and—most importantly—improved the overall mortality of rats with endotoxemia [23]. These functional improvements in ALI were associated with attenuated endogenous iNOS expression in animals pretreated with isoflurane [23]. In the representative Western blots, iNOS expression was actually enhanced in the lung homogenate of sham-operated animals receiving isoflurane, although the difference was blunted by the overexpressed iNOS protein level in the control animals with ALI (Fig. 6, [23]).

Our findings are substantially supported by these closely relevant reports. Nevertheless, our study first speculates the molecular mechanism of preconditioning pulmonary protective effect of isoflurane, and this response is mainly mediated through activation of endogenous iNOS in the lung. We speculated a major drawback in our study design was that the respiratory physiology of animals receiving isoflurane treatment might be suppressed by a volatile agent. Therefore, the experimental outcomes could be

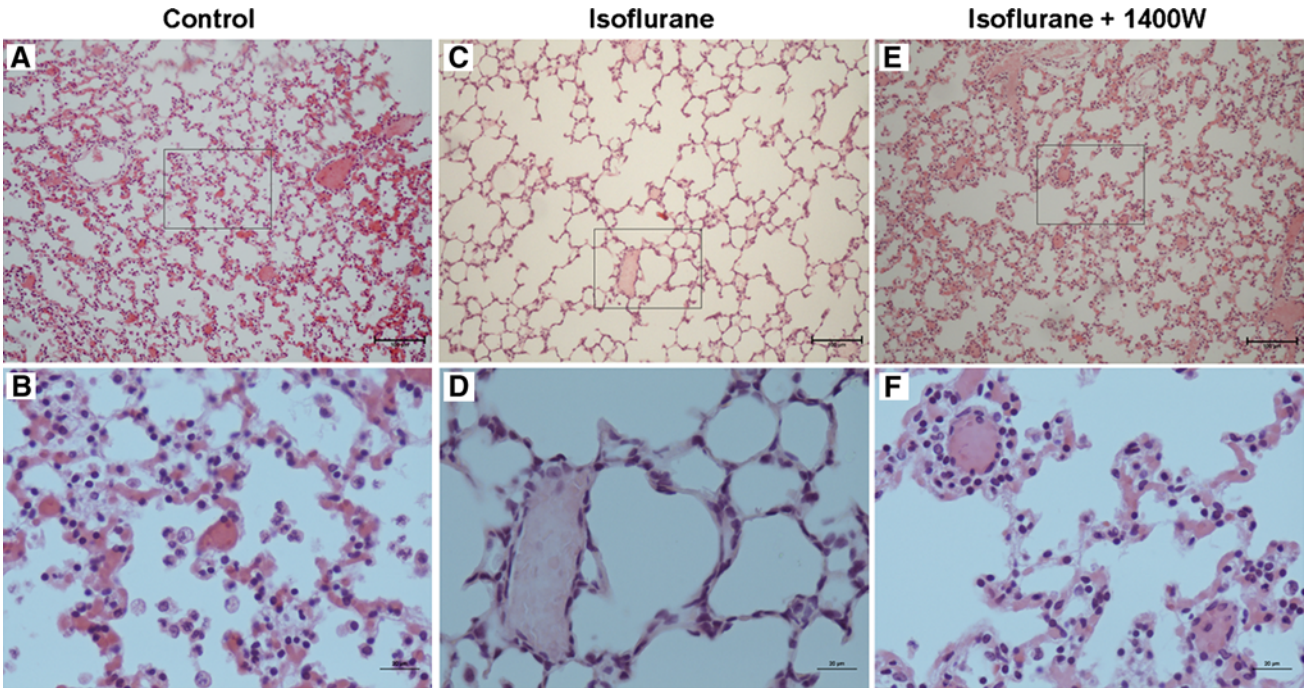

Fig. 7 Degrees of endotoxin-induced injury were assessed by histological changes in lung sections under light microscopy. Formation of eosinophilic hyaline material lining alveolar units and infiltration of inflammatory cells were ameliorated in the lung sections of animals receiving isoflurane preconditioning. Pretreatment with a selective inducible nitric oxide synthesis (iNOS) inhibitor (1400W) diminished the pulmonary protective effect of isoflurane, evidenced by increased inflammatory infiltration and hyaline membrane formation. **a**, **b** Representative lung sections of control animals; **c**, **d** representative lung sections of isoflurane-only group; **e**, **f** representative lung sections of isoflurane + 1400W group. All animals received intratracheal instillation of lipopolysaccharide

affected by the development of hypercapnic acidosis in the isoflurane-treated group. Hypercapnic acidosis has been shown to attenuate degrees of ALI and systemic septicemia [24, 25]. In our opinion, hypercapnia-mediated lung protective response is a therapeutic approach during the development of ALI, and the pulmonary protection of isoflurane is mediated via the pharmacologic preconditioning mechanism. The 3-h washout period after isoflurane exposure would thus minimize the effect of hypercapnic acidosis in our study.

Preconditioning is a phenomenon characterized by therapeutic management and precedes sustained permanent tissue damage, resulting in improvement of organ dysfunction. The molecular mechanisms of anesthetic preconditioning are multimodal, including activation of endogenous G-protein-coupled receptors, protein kinases, adenosine triphosphatase (ATP)-sensitive potassium channel, and iNOS [26]. Generation of sublethal levels of NO and reactive oxygen species during the initiation of preconditioning activates phospholipase C and protein kinase C (PKC), and in turn amplifies the preconditioning stimulus [26]. In fact, iNOS-mediated preconditioning protective effect of halogenated anesthetics has been manifested in other organs, such as neuronal and myocardial systems [27, 28]. In addition, it is also essential to compare the preconditioning pulmonary protective effect of different volatile anesthetics, as airway responsiveness differs among these anesthetic agents.

In conclusion, administration of volatile isoflurane before induction of experimental ALI provides preconditioning pulmonary protective effect against endotoxin-induced lung injury, mainly by suppressing anti-inflammatory response in the lung. Fundamentally, our study provides mechanistic insight into the preconditioning pulmonary protective effect of isoflurane in an experimental model of ALI, and this effect is cardinally mediated through activation of endogenous iNOS in the lung.

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