Sevoflurane attenuates ventilator-induced lung injury by regulating c-PLA₂ expression

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Abstract. The aim of the present study was to investigate the potential role of club cell secretory protein (CCSP), an endogenous modulator, in reducing pulmonary inflammation induced by sevoflurane following one-lung ventilation (OLV). Healthy Japanese white rabbits were randomly assigned to six groups: Sham-operated group (group S); respiratory management of OLV group (group O); OLV + sevoflurane treated group (group OF), club cells exfoliated + sham-operated group (group NA), club cells exfoliated + OLV group (group NAO); and club cells exfoliated + OLV + sevoflurane treated group (group NAOF). At the end of the experimental observation, all animals in the different groups were sacrificed and lung injury was evaluated according to the lung wet/dry weight ratio and histological scoring system. Lung homogenates were harvested to detect the mRNA and protein expression of cytosolic phospholipase A2 (c-PLA₂) and CCSP. The content of arachidonic acid was measured using an ELISA. Following OLV treatment, c-PLA₂ expression was increased, CCSP expression was decreased and lung injury scores were significantly increased. Sevoflurane inhalation in the OLV-treated group induced an upregulation of CCSP and a downregulation of c-PLA₂ expression. In the group NAO, in which the club cells were simultaneously exfoliated, OLV caused more severe lung damage and induced higher expression of c-PLA₂ compared with that in group O.

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Abbreviations: AA, arachidonic acid; ALI, acute lung injury; CCSP, club cell secretory protein; c-PLA₂, cytosolic phospholipase A₂; OLV, one lung ventilation; PIPs, peak inspiratory pressure; PLA₂, phospholipase A₂; SVF, sevoflurane; W/D, wet-to-dry lung weight

Key words: sevoflurane, one-lung ventilation, lung inflammation, club cell secretory protein, cytosolic phospholipase A2

However, sevoflurane inhalation reduced the extent of lung injury and the expression of c-PLA₂, even when the endogenous modulator of lung inflammation, CCSP, was exfoliated (group NAOF). These results indicated that OLV promoted lung inflammation through the CCSP and c-PLA₂ pathway. However, the results from the club cells exfoliated group indicated that the CCSP may not be involved in the protective effect exerted by sevoflurane inhalation.

Introduction

In 1984, Zeldin *et al* (1) reported 10 cases of pulmonary complications following pneumonectomy under one-lung ventilation (OLV). Since then, it has been well established that the mechanisms of OLV-induced acute lung injury (ALI) are similar to those of ventilator-induced lung injury, in which inflammatory responses serve a key role (2-4). Clinically, there is no specific measure to prevent ALI induced by mechanical ventilation, apart from certain protective ventilation strategies, including using variable tidal volumes, tidal volume <8 ml/kg predicted body weight, pressure control ventilation, and peak inspiratory pressure (PIP) <35 cm H₂O (4). However, animal studies have demonstrated that, even with protective ventilation measures, inflammatory responses still occurred (5). Therefore, it may be hypothesized that inhibition of inflammation responses may alleviate lung injury induced by OLV.

Phospholipase A₂ (PLA₂), a key rate-limiting enzyme, catalyzes the hydrolysis of membrane phospholipids to lysophospholipids, platelet-activation factor and arachidonic acid (AA) (6). Group IVA cytosolic c-PLA₂ (GIVA c-PLA₂; molecular weight 85 kDa), which preferentially hydrolyzes AA at the sn-2 position of phospholipids, is a member of the large c-PLA₂ family (7,8). Currently, GIVA c-PLA₂ is generally considered to be a key enzyme mediating in the generation of multiple lipid mediators, including the agonist-induced release of AA for eicosanoid production, and is recognized as a potentially important pharmacological target for the control of inflammatory diseases (9). The downstream products of AA and its metabolism possess the potential to increase vascular permeability, which may lead to inflammatory responses (10,11). The inhibition of cPLA₂ may attenuate the OLV-induced acute lung injury by reducing the downstream products in AA metabolism, which is responsible for the

increases in vascular permeability, the recruitment of neutrophils and cell injury (12).

Club cell secretory protein (CCSP) is secreted by non-ciliated club cells (formerly referred to as Clara cells) into the bronchial epithelium of the mammalian lung. As a biomarker of club cells and lung health, CCSP has been used as a useful diagnostic marker for toxicant exposure or airway epithelial damages (13). Mice deficient in CCSP developed more prominent lung injury and inflammatory responses following exposure to oxidant treatment or virus challenge, which indicated that CCSP acts as an endogenous inhibitor of PLA₂ (14,15).

Sevoflurane (SVF) is widely used in the induction and maintenance of general anesthesia. A number of studies demonstrated that SVF has anti-inflammation properties and it is also involved in the protective mechanisms against ischemia/reperfusion injury (16,17). A previous study demonstrated that, in OLV-induced acute lung injury model, SVF inhalation significantly reduced the lung injury decreased c-PLA₂ expression levels, lung wet-to-dry (W/D) weight ratios and histological scores in a dose-dependent manner. However, SVF inhalation exerted no significant effect on the expression of CCSP (18). These results suggested that the protective role of SVF against OLV-induced acute lung injury may be associated with c-PLA₂ regulation, but not with CCSP. In the present study, club cell-exfoliated rabbits were used to determine whether SVF attenuates pulmonary inflammation via c-PLA₂ downregulation, rather than CCSP regulation.

Materials and methods

Ethics approval. The present study complied with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (19). All experimental procedures were approved by the Animal Ethics Committee and Research Ethics Committee of Yunnan Province, China (license no. KM3476-821), and Kunming Medical University (Kunming, China; license no. KY976222-m23).

Animal preparation. The present study strictly abided by the guidelines of Kunming Medical University for the care and use of laboratory animals. A total of 36 healthy Japanese white rabbits (weight, 2.2-2.5 kg) of either sex (1:1 male:female) were purchased from the Experimental Animal Center of Kunming Medical University [Animal Certificate of Conformity no. 0020946; animal license no. scxk (Yunnan) 2011-0004]. The rabbits were randomly divided into six groups (n=6 per group): Sham-operated group (group S), OLV group (group O), OLV+SVF inhalation group (group OF), club cells exfoliated + sham-operated group (group NAO) and club cells exfoliated + OLV sevoflurane group (group NAOF).

In group S, the trachea, left common carotid artery and right external jugular vein were exposed, followed by placing an endotracheal tube (inner diameter of 2.0 mm) via tracheotomy between tracheal rings 2 and 3, without further surgery. OLV was achieved by placing the tracheotomy tube into the right main bronchus. Mechanical ventilation was performed by OLV for 2 h followed by two-lung ventilation (TLV) for 1 h with an identical ventilator (Aestiva/5 7900; Datex Ohmeda Inc.; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The setting parameters were as for groups that received OLV were as follows: Inspired oxygen fraction, 1.0; tidal volume, 20 ml/kg; respiration rate, 30 breaths/min; and inspiratory:expiratory ratio, 1:2. SVF (2.5% vol) was administered during mechanical ventilation by ventilator (Aestiva/5 7900, as above; group OF and NAOF). For animals in the group NA, exfoliating club cells were created by exposure to naphthalene vapor at a concentration of 100 mg/l for 12 h.

Anesthesia and intraoperative detections. The mean arterial pressure, end tidal CO_2 and SVF concentrations were continuously monitored and maintained within normal ranges. Ringer's solution (10 ml/kg/h) was continuously infused through the right external jugular vein catheter. The rabbits were anesthetized with pentobarbital sodium (30 mg/kg intravenously; Shandong West Asia Chemical Industry Co., Ltd., Shandong, China) during the sham surgeries. Anesthesia was maintained by continuous infusion of remifentanil (cat. no. 1110710; Yichang Humanwell Pharmaceutical Co., Ltd., Yichang, China) at a rate of 1 μ g/kg/min and intermittent administration of vecuronium (0.1 mg/kg per 30 min; cat. no. 111004.2; Zhejiang Xianju Pharmaceutical Co., Ltd., Zhejiang, China) during either OLV or TLV.

Lung W/D ratio evaluation. The right lower lobe was excised and the wet weight was recorded. The lobe was then desiccated at 80° C for 72 h to measure the dry weight. The W/D ratio was calculated.

Lung histological score. The right upper lobe were fixed for 6-12 h in 4% paraformaldehyde at 20-24°C, embedded in paraffin wax and then cut into 5 μ m sections using a microtome. The sections were stained using a hematoxylin and eosin staining kit (cat. no. G1120; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 20-24°C according to the manufacturer's instructions. In brief, sections were stained in hematoxylin (1 g/l) for 16 min and eosin (1 g/100 ml) for 15 sec. Slides were viewed by a pathology technician using light microscopy for histological evaluation of the lung injury.

Quantification of CCSP and c-PLA₂ mRNA expression levels. Total RNA was extracted from the right middle lobes using a TRIzol RNA Extraction kit (cat. no. 12183555; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The first-strand cDNA was synthesized using Superscript IV Reverse Transcriptase system (cat. no. 18091050; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, 50 μ M Oligo(dT)20 and 10 mM dNTP mix was added into 1.0 μ g total RNA and annealed at 65°C for 5 min. Following this, 200 U/ μ l Superscript IV reverse transcriptase and 100 mM DTT was added in the presence of SSIV buffer, and the RT system was incubated for 10 min at 55°C, followed by 10 min at 80°C. Reverse transcription-quantitative polymerase chain reaction was performed using a BioEasy SYBR Green I Real Time PCR kit (cat. no. 04913850001; Roche Diagnostics GmbH) according to the manufacturer's protocols. The reaction took place at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The experiment was replicated



Figure 1. Effects of OLV and sevoflurane inhalation on the expression levels of c-PLA₂ and CCSP mRNA in rabbit lung. Relative expression levels of (A) c-PLA₂, and (B) CCSP quantified by reverse transcription-quantitative polymerase chain reaction. $^{\circ}P<0.05$ vs. S; $^{\bullet}P<0.05$ vs. O; $^{*}P<0.05$ vs. NA; $^{\circ}P<0.05$ vs. NAO. OLV, one-lung ventilation; c-PLA₂, cytosolic phospholipase A₂; S, sham-operated group; O, OLV group; OF, OLV + sevoflurane group; NA, club cells exfoliated + sham-operated group; NAO, club cells exfoliated + OLV group; NAOF, club cells exfoliated + OLV + sevoflurane group; CCSP, club cell secretory protein.

three times per sample. The results were quantified using the $2^{-\Delta\Delta Cq}$ method (20). The forward and reverse sequences for the primers (5'-3') used in this study were as follows: β -actin, forward CATCCTGACGCTCAAGTA, reverse GTTGTA GAAGGTGTGGTG; CCSP, forward CACCAAAGCCTC CAACCT, reverse GGCAGATGTCCGAAGAAG; c-PLA₂, forward CCTAATCATTGTTGGAAGATAA, reverse ATG GTTGCTTGGAGAATA.

Western blotting. Freshly frozen samples of lung tissues from the rabbits were homogenized in pre-cooled radioimmunoprecipitation assay lysis buffer (cat. no. P0013C; Beyotime Institute of Biotechnology, Haimen, China) supplemented with 15 μ l protease inhibitor cocktail (cat. no. 5892970001; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The protein concentrations were determined by using a bicinchoninic acid kit (cat. no. P0010; Beyotime Institute of Biotechnology). Proteins (80 μ g/lane) were separated by 10% SDS-PAGE. The membrane was blocked with 5% bovine serum albumin (cat. no. V900933; Sigma-Aldrich; Merck KGaA) at 20-24°C for 1.5 h. GAPDH was used as the reference gene. For western blotting, the nitrocellulose membranes were incubated overnight at 4°C with primary antibodies against CCSP $(2 \mu g/ml; cat. no. ab50711; Abcam, Cambridge, UK), c-PLA_2$ $(1 \ \mu g/m)$; cat. no. ab73406; Abcam) and GAPDH (1:2,000; cat. no. 20011; Abmart, Inc., Shanghai, China), followed by incubation with the secondary antibody (cat. no. M21003; Abmart) at room temperature for 2 h. The blots were detected by enhanced chemiluminescence (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the band intensities were quantified using ImageJ software 1.44 (National Institutes of Health, Bethesda, MA, USA).

ELISA. The content of AA in lung tissues was measured using a commercially available sandwich ELISA kit (cat. no. CSB-EQ027590RB; Wuhan Huamei Biotech Co., Ltd., Wuhan, China) according to the manufacturer's protocols.

Statistical analysis. The quantitative data are presented as the mean \pm standard error of the mean. One-way analysis of variance (with repeated measures) followed by Fisher's least significant difference post hoc test was used as indicated for comparisons



Figure 2. Effects of OLV and sevoflurane inhalation on the expression levels of c-PLA₂ and CCSP proteins in rabbit lung. c-PLA₂ and CCSP protein expression was evaluated by western blot analysis. c-PLA₂, cytosolic phospholipase A₂; S, sham-operated group; O, OLV group; OF, OLV + sevoflurane group; NA, club cells exfoliated + sham-operated group; NAO, club cells exfoliated + OLV group; NAOF, club cells exfoliated + OLV + sevoflurane group; CCSP, club cell secretory protein.

between the groups. SPSS v18.0 (SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

*Effects of sevoflurane on the expression levels of c-PLA*₂ *and CCSP.* In the group O, c-PLA₂ expression was significantly increased compared with the sham group (Fig. 1A), whereas CCSP expression was decreased following OLV (P<0.05 vs. group S; Fig. 1B). Following OLV, SVF inhalation (group OF) significantly reduced c-PLA₂ expression (Fig. 1A), and CCSP expression increased (vs. group O; Fig. 1B).

The effects of SVF on the expressional changes of CCSP, an endogenous inhibitor of PLA₂, was further analyzed using the club cell-exfoliated model exposed to naphthalene vapor at a concentration of 100 mg/l for 12 h (groups NA, NAO and NAOF). Low expression levels of CCSP were detected at the mRNA and protein level in groups NA, NAO and NAOF, indicating that club cells were successfully exfoliated (Figs. 1B and 2). As the endogenous inhibitor of c-PLA₂ was not present, the expression of c-PLA₂ was the highest in group NAO. However, following SVF inhalation in the group NAOF, the expression of c-PLA₂ was significantly reduced compared with group NAO (P<0.05; Fig. 1A). A similar pattern of c-PLA₂ and CCSP protein expression was observed in the lungs of the three groups of rabbits (Fig. 2).



Figure 3. Association of lung injury following OLV and sevoflurane inhalation in rabbit lung. (A) Arachidonic acid was determined using ELISA. (B) Lung wet-to-dry-weight ratios following OLV or OLV and sevoflurane inhalation in either normal rabbits or club cells exfoliated rabbits. (C) Histological lung injury scores in all experimental groups. $^{+}P<0.05$ vs. S; $^{+}P<0.05$ vs. O; $^{+}P<0.05$ vs. NA; $^{+}P<0.05$ vs. NAO. OLV, one-lung ventilation; AA, arachidonic acid; S, sham-operated group; O, OLV group; OF, OLV + sevoflurane group; NA, club cells exfoliated + sham-operated group; NAO, club cells exfoliated + OLV group; NAOF, club cells exfoliated + OLV sevoflurane group; W/D, wet-to-dry weight.

Evaluation of pulmonary AA content, W/D ratio and histological scores. Pulmonary AA was determined using an ELISA kit (Fig. 3A). No significant difference in AA content was observed between group S and group NA. The concentrations of AA were significantly increased in all groups following OLV, compared with group S and NA. As expected, the AA contents was reduced following SVF inhalation in the groups OF, NA and NAOF, compared with the groups O and NAO (Fig. 3A). The W/D ratio was used as an indicator of edema formation following OLV. The data revealed that the W/D ratio did not differ significantly between groups S and NA. The W/D ratio increased by 13.3% in group O compared with that in group S. SVF inhalation reduced the W/D ratio in group OF compared with in group O (Fig. 3B). The highest W/D ratio was observed in the group NAO, while SVF inhalation reduced the W/D ratio in the group NAOF compared with group NAO. The histological lung injury scores of different groups are presented in Fig. 3C. OLV increased lung injury scores by 8 times in the O group compared with S group, and 10 times in the NAO group compared with NA group. Following SVF inhalation, the histological lung injury scores were significantly reduced in groups OF and NAOF, compared with in groups O and NAO, respectively.

Assessment of lung histopathological changes. On examining hematoxylin and eosin-stained lung tissue sections, no significant pathological changes were observed in the sham-operated group (group S) and club cells exfoliated + sham-operated group (group NA; Fig. 4). OLV caused severe lesions in the group O, manifesting as lung hyperemia and hemorrhage, alveolar wall thickening and increased exudation, with significant infiltration of red blood and inflammatory cells in the alveolar spaces. The pathological changes in the group NAO were more extensive compared with those in the group O. In groups OF and NAOF, those in the pathological lesions of lung tissues were significantly alleviated compared with group O and NAO, respectively. However, the pathological lesions in group NAOF were more pronounced compared with those in group OF.

Discussion

In the present study, SVF inhalation was demonstrated to attenuate OLV-induced ALI, which was associated with c-PLA₂ downregulation by regulating the endogenous inhibitor of PLA₂. With the advances in thoracic surgery techniques, OLV has been routinely used to facilitate surgical exposure; however, OLV further increases lung injury and leukocyte recruitment, independent of the administration of propofol or desflurane anesthesia (21,22).

 PLA_2 is a key enzyme that hydrolyzes membrane phospholipids. It serves a crucial role in pulmonary inflammation through modulating membrane signal transduction, membrane stability and formation of leukocyte-endothelial cell



Figure 4. Effects of OLV and sevoflurane inhalation on OLV-induced lung inflammation. Histological examination of a lung section by hematoxylin and eosin staining. OLV, one-lung ventilation; H&E, hematoxylin and eosin; S, sham-operated group; O, OLV group; OF, OLV + sevoflurane group; NA, club cells exfoliated + sham-operated group; NAO, club cells exfoliated + OLV group; NAOF, club cells exfoliated + OLV + sevoflurane group.

adhesion (23). c-PLA₂ is an 85-kDa protein, widely distributed in cells, and it is activated by phosphorylation through the mitogen-activated protein kinase pathway during ventilation to release AA (12,24,25). AA is further metabolized to generate inflammatory mediators, including prostaglandins and thromboxanes. Therefore, inhibition of c-PLA₂ may alleviate ventilation-induced lung injury by reducing these AA downstream products, which cause edema during acute lung inflammation (26). CCSP is an endogenous modulator of lung inflammation. In vitro, CCSP inhibits the activation of c-PLA₂ by binding Ca²⁺, a cofactor of c-PLA₂ activation (6). A previous study demonstrated that after 2 and 4 h high-PIP ventilation in CCSP^{-/-} mice, the W/D ratios were significantly higher compared with those in wild-type mice, indicating a greater vascular permeability increase in CCSP^{-/-} mice (27). This evidence was consistent with the results of the present study, which demonstrated that OLV indeed caused lung inflammation with evidence of upregulated c-PLA₂, higher AA concentration and higher W/D ratios. It is hypothesized that the upregulated c-PLA₂ in group O may be associated with the activation of nuclear factor-kB and the production of pro-inflammatory cytokines induced by OLV. In the NA, NAO and NAOF groups, CCSP was reduced compared with groups S, O and OF, as the club cells were damaged by exposure to naphthalene vapor (club cells exfoliated). Thus, the expression of $c-PLA_2$ in the NAO group was increased compared with that in the O group.

Clinical and experimental research has demonstrated that inhalation of SVF may reduce inflammation in patients with ALI (28-30). SVF is a widely used anesthetic agent, and the protective effect of SVF under conditions of hypoxia or endotoxemia has been extensively studied (31). Rodríguez-González *et al* (32) suggested that SVF helped to preserve endothelial integrity in severe endotoxemia. Shen *et al* (30) also demonstrated that repeated SVF inhalation significantly reduced airway inflammation in the lungs of allergic mice. Therefore, the ability of SVF to act protectively against inflammation expands its therapeutic value beyond its anesthetic properties.

In the present study, it was observed that lung injury was alleviated following SVF inhalation in animals with OLV-induced-acute lung injury. SVF inhalation also reduced the expression of c-PLA₂, the lung W/D ratio and histological scores in a concentration-dependent manner. However, the expression of CCSP exhibited no significant changes. These results are notable as CCSP is a well-established endogenous modulator of c-PLA₂. In the current study, a club cell exfoliated rabbit model was also used to investigate the potential association between SVF, c-PLA₂ and CCSP. Little CCSP expression was detected in the club cell exfoliated model rabbits, which indicated that the club cells were successfully exfoliated. Furthermore, no significant changes were observed between the groups S and NA. The data indicated that exfoliation of club cells may not cause lung inflammation. The data revealed that, in club cell exfoliated animals, the expression of c-PLA₂, the concentration of AA, the W/D ratio and the lung injury scores were significantly decreased in group NAOF compared with group NAO. The results suggested that the protective role of SVF under OLV may not be associated with CCSP. Other inflammation modulators may participate in the SVF signaling pathway; however, further studies are required.

In conclusion, the results of the present study indicated that OLV caused lung inflammation through CCSP and $c-PLA_2$ regulation. The inhalation of SVF reduced lung inflammation in a CCSP-independent manner.

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Availability of data and materials

The datasets generated and analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

YY, WFW and RL wrote and critically revised the manuscript. YHL and LSL performed the animal experiments. XG, YHL and RL performed the PCR, ELISA and western blot analyses. RL was in charge of the design and conception of the manuscript. YY and WFW performed HE stain and lung histopathological assessment. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study complied with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental procedures were approved by the Animal Ethics Committee and Research Ethics Committee of Yunnan Province (license no. KM3476-821) and Kunming Medical University (license no. KY976222-m23).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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