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Isoflurane, but Not Sevoflurane, Increases Transendothelial Albumin Permeability in the Isolated Rat Lung

Role for Enhanced Phosphorylation of Caveolin-1

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Background: **Caveolae-mediated transendothelial transport of albumin has recently been shown to be the primary mechanism regulating microvascular endothelial albumin permeability. The authors investigated the effects of isoflurane and sevoflurane on pulmonary endothelial albumin permeability and assessed the potential role of the caveolae scaffold protein, caveolin-1, in these effects.**

Methods: **Isolated rat lungs and cultured rat lung microvessel endothelial cells (RLMVECs) were exposed to 1.0 or 2.0 minimum alveolar concentration (MAC) isoflurane or sevoflurane for 30 min. ^{125}I-albumin permeability–surface area product and capillary filtration coefficient were determined in the isolated lungs. In RLMVECs, uptake and transendothelial transport of ^{125}I-albumin were measured in the absence and presence of pretreatment with 2 mM methyl-β-cyclodextrin, a caveolae-disrupting agent. Uptake of fluorescent-labeled albumin, as well as phosphorylation of *Src* kinase and caveolin-1, was also determined. In Y14F-caveolin-1 mutant (nonphosphorylatable) expressing RLMVECs, uptake of ^{125}I-albumin and phosphorylation of caveolin-1 were evaluated.**

Results: **In the isolated lungs, 2.0 MAC isoflurane increased ^{125}I-albumin permeability–surface area product by 48% without affecting capillary filtration coefficient. In RLMVECs, isoflurane more than doubled the uptake of ^{125}I-albumin and caused a 54% increase in the transendothelial transport of ^{125}I-albumin. These effects were blocked by pretreatment with methyl-β-cyclodextrin. The isoflurane-induced increase in uptake of ^{125}I-albumin in wild-type RLMVECs was abolished in the Y14F-caveolin-1 mutant expressing cells. Isoflurane also caused a twofold increase in *Src* and caveolin-1 phosphorylation. Neither 1.0 MAC isoflurane nor 1.0 or 2.0 MAC sevoflurane affected any index of albumin transport or phosphorylation of caveolin-1.**

Conclusion: **Isoflurane, but not sevoflurane, increased lung transendothelial albumin permeability through enhancement of caveolae-mediated albumin uptake and transport in the isolated lung. This effect may involve an enhanced phosphorylation of caveolin-1.**

CAVEOLAE are flask-shaped invaginations of the plasma membrane and free cytoplasmic plasmalemmal vesicles that are involved in endocytosis, signal transduction, mechanotransduction, potocytosis, and cholesterol trafficking.[1] Endothelial caveolae have been demonstrated to regulate capillary permeability by their participation in the process of transcytosis, the primary means of albumin transport across continuous endothelia.[2,3] This pathway is thought to regulate the oncotic pressure gradient across the microvessel wall (with its impact on fluid distribution) and mediate the delivery of albumin-bound hormones and drugs into the underlying tissue. Caveolin-1, an integral membrane protein (20–22 kd), is the specific marker and major structural component of endothelial caveolae.[4] Evidence has accumulated suggesting that caveolin-1 regulates endothelial uptake and transcellular transport of albumin.[2] Caveolin-1 null mice were shown to be devoid of caveolae and defective with respect to uptake and transport of albumin, which could be reversed by introduction of caveolin-1 complementary DNA.[5–7]

Evidence has accumulated from animal models[8–10] suggesting that exposure to a volatile anesthetic can increase the permeability of the alveolar–capillary barrier and decrease the threshold for pulmonary edema. In humans, isoflurane anesthesia was associated with an increase in pulmonary clearance of ^{99m}Tc DTPA, which implied an enhanced permeability of the alveolar–capillary barrier.[11] The contribution of caveolae-mediated albumin uptake and transport to the increase in permeability of the alveolar–capillary barrier caused by volatile anesthetics is unknown and remains to be determined.

The goal of this study was to evaluate the effects of isoflurane and sevoflurane on pulmonary endothelial albumin permeability. After we found that isoflurane increased endothelial albumin permeability in the isolated, perfused rat lung, mechanistic information was obtained by assessing changes in albumin uptake and transport in

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rat lung microvascular endothelial cells (RLMVECs) in the absence and presence of pretreatment with methyl-β-cyclodextrin, a caveolae disrupting agent. The role of caveolin-1 in the effects of volatile anesthetics on endothelial permeability was evaluated by Western blot analysis and by comparing changes in wild-type pulmonary endothelial cells *versus* phosphorylation-defective Y14F-caveolin-1 mutant expressing RLMVECs.

Materials and Methods

The study was performed in the isolated rat lungs and cultured rat lung microvascular endothelial cells.

Studies in Isolated Lungs

Isolated Lung Preparation. After approval from the Institutional Animal Care and Use Committee (Chicago, Illinois), studies were conducted in 35 adult Sprague-Dawley rats (Charles River, Wilmington, MA) of either sex (300–350 g). The rats were anesthetized with pentobarbital sodium (40 mg/kg intraperitoneal). After tracheal intubation, the lungs were mechanically ventilated with room air at a rate 60 breaths/min and a tidal volume of 2.8 ml. A midsternal thoracotomy was performed, and 400 U heparin was injected into the vena cava for anticoagulation. The pulmonary artery and left atrium were cannulated and the aorta was ligated so that the venous effluent could be drained into a left atrial cannula. The heart and lungs (with a segment of the trachea) were removed *en bloc* from the thoracic cavity and mounted on a perfusion apparatus. The lungs were immediately perfused with Krebs solution at 37°C *via* the pulmonary artery at a constant flow (0.035 ml/g) and venous pressure (−4.0 cm H_2O). The composition of the solution was as follows: 118 mM NaCl, 4.7 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM HEPES, 11 mM glucose, and 0.025 mM EDTA (pH 7.35–7.45) supplemented with bovine serum albumin (3 g/100 ml). A continuous record of pulmonary arterial pressure and lung weight was obtained *via* an in-line pressure transducer and a counterweighted beam balance during experiments. These data were displayed on a personal computer video monitor (IBM PS2 model 50z; White Plains, NY) after being digitized by an analog-to-digital converter (μDAS 8PGA board; Keithley Metrabyte, Solon, OH). Data acquisition software (Notebook Pro for Windows; Labtech, Andover, MA) was used to analyze data. The lungs were ventilated at a constant peak inspiratory pressure (5 cm H_2O) throughout and end-expiratory pressure of 2 cm H_2O.[12]

After a stabilization period of 15 min, the isolated lung was ventilated with room air (carrier gas) alone (control) or with 1.0 and 2.0 minimum alveolar concentration (MAC) isoflurane (1.4% and 2.8%) or sevoflurane (2.4% and 4.8%)[13,14] in air by means of a calibrated vaporizer (Dräger, Lübeck, Germany) for 30 min. Inspired concentrations of isoflurane and sevoflurane were confirmed by gas chromatography.

Permeability–Surface Area Product. Albumin permeability–surface area product (PS) product was determined using the single-sample technique.[15,16] After 30 min of administration of either carrier gas or anesthetic, the isogravimetric lung preparation was rapidly switched to perfusate, which contained tracer ^{125}I-albumin (approximately 80,000 counts/ml) and 0.3 g/100 ml unlabeled albumin. After 3 min of exposure to ^{125}I-albumin, the lung was switched back to buffer without tracer but with 3.0 g/100 ml albumin for 6 min to remove cell surface and circulating ^{125}I-albumin, and then the perfusion was abruptly stopped. The lungs were immediately removed, cleaned of connective tissue, weighed, and counted for γ radioactivity. Samples of the instilled ^{125}I-albumin were also counted. PS product (in $ml \cdot min^{-1} \cdot g^{-1}$ dry lung) was calculated with the formula $A/(Cp \cdot t)$, where A and Cp are concentrations of tracer albumin in the tissue (in counts/g) and in the perfusate (in counts/ml), respectively, and t is the perfusion time for tracer albumin (3 min).[15–17] Previous studies indicated that a 3-min infusion period was sufficient for ^{125}I-albumin to flux across the endothelium.[15,16] With this protocol, back flux of ^{125}I-albumin from the pulmonary interstitium could be considered negligible because of the transient infusion of the labeled albumin.[15] Therefore, the accumulation of ^{125}I-albumin is taken as a unidirectional protein flux across the endothelial barrier into the interstitium.

Capillary Filtration Coefficient. Capillary filtration coefficient ($K_{f,c}$) was determined in separate lung preparations using a method described previously.[12] Before and after 30 min of administration of either carrier gas or anesthetic, the $K_{f,c}$ was measured from the rate of lung wet weight gain more than 1 min after a step increase (+6 cm H_2O) in venous pressure for 5 min. The rate of weight gain was normalized by the lung dry weight and step size to calculate $K_{f,c}$. The analytical procedures used for computing $K_{f,c}$ from recordings of lung wet weight have been presented in detail.[18] Values are expressed as $ml \cdot min^{-1} \cdot cm\ H_2O^{-1} \cdot g^{-1}$ dry lung tissue.

Wet/Dry Lung Weight Ratio. Wet/dry lung weight ratio served as an index of lung water content. For determination of lung dry weight, lung tissue was dried in an oven to a constant weight (60°C for 72 h).

Studies in Cultured Pulmonary Endothelial Cells

Endothelial Cell Culture. Rat lung microvascular endothelial cells were obtained from Vec Technologies (Rensselaer, NY). For monolayer culture, the cells were resuspended at a density of 1×10^5/ml in high-glucose Dulbecco's modified Eagle's medium (supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin) and incubated (37°C) under a hu-

midified atmosphere of 5% CO_2–95% air. Confluent monolayers formed on culture dishes, microporous Transwell filter inserts, or glass coverslips within 24–48 h. RLMVECs stably expressing Y14F-caveolin-1 were generated as described previously.[3,19] RLMVEC monolayers were serum deprived for 2 h before experiments.

Anesthetic Delivery System. The 6-well or 12-well cell culture plates containing RLMVEC monolayers in HEPES-buffered Hanks' balanced salt solution (HBSS, 1.0 or 0.5 ml/well, respectively) were placed in a layer of water within the airtight exposure chamber in a water bath heated to 37°C.[20] Carrier gas (air) was passed through a calibrated vaporizer (Dräger, Lübeck, Germany) providing 1.0 and 2.0 MAC of either isoflurane (1.4% and 2.8%) or sevoflurane (2.4% and 4.8%) at a flow rate of 5 l/min into the airtight chamber for 30 min. Preliminary experiments showed that complete equilibration of anesthetics between the liquid (HBSS) and gas phases was achieved within 2 min. The concentrations of volatile anesthetics were confirmed by sampling on both the inflow and the outflow side of the chamber and in the wells of the multiwell plates and assessing the anesthetic concentration by gas chromatography (mode 5890; Hewlett Packard, Wilmington, DE).

Uptake of ^{125}I-Albumin. Uptake of ^{125}I-albumin into wild-type RLMVECs was assayed as described previously,[21] with minor modifications. Briefly, confluent RLMVEC monolayers were seeded in six-well plates (1.0–1.2 $\times$ 10^6cells/35 mm). Unlabeled albumin was added at a final concentration of 0.1 mg/ml in four wells and 100 mg/ml in other two wells containing HBSS, respectively. ^{125}I-albumin was then added into each well at an activity of 1 $\times$ 10^6 counts per minute (cpm). Uptake of ^{125}I-albumin was allowed to proceed at 37°C for 30 min during exposure to either carrier gas (air) or 1.0 and 2.0 MAC isoflurane or sevoflurane. Albumin uptake was terminated by chilling the samples on ice. All samples were then washed three times in acid wash buffer (0.5 M NaCl and 0.2 M acetic acid, pH 2.5) and another three times in cold HBSS, to remove any ^{125}I-albumin that attached to the cell surface. Cells were finally lysed with 1 ml Tris-HCl buffer (0.05 M Tris-HCl, 1% Triton X-100, and 0.5% SDS, pH 7.4). The radioactivity in samples was determined using a γ counter (Packard Instruments, Downers Grove, IL). Specific uptake of ^{125}I-albumin was estimated by subtracting the nonspecific cell-associated activity (determined in the presence of 100 mg/ml unlabeled albumin) from the total (determined in the presence of 0.1 mg/ml unlabeled albumin).[21] Specific uptake was normalized to cell protein (determined by bicinchoninic acid method) and expressed in units of cpm/mg cell protein.

On the basis of our initial experimental results, additional experiments, using the same basic protocol, were performed to evaluate the roles of the caveolae and caveolin-1 in the effect of 2.0 MAC isoflurane on albumin uptake. In one group of studies, endothelial cells were pretreated with methyl-β-cyclodextrin, the cholesterol-binding agent that disassembles caveolae.[21,22] This pretreatment consisted of incubation of RLMVECs with 2.0 mM methyl-β-cyclodextrin in HBSS for 15 min at 37°C, followed by two washes with HBSS. In the second group of studies, Y14F-caveolin-1 mutant RLMVECs were used.

Determination of Fluorescent Albumin Uptake by Confocal Microscopy. To confirm the effect of volatile anesthetic on uptake of ^{125}I-albumin, we performed studies on fluorescent albumin uptake in RLMVECs. For determinations of fluorescent albumin uptake into RLMVECs, we used confluent RLMVEC monolayers grown on glass coverslips. Monolayers were treated with fluorescently tagged albumin (Alexa 488-labeled bovine serum albumin, at 50 μg/ml, plus 0.5 mg/ml unlabeled albumin in HBSS) and simultaneously exposed to air (control) or 2.0 MAC isoflurane or sevoflurane for 30 min at 37°C.[21] Unincorporated probe was removed by rinsing with HBSS.

Internalized fluorescent albumin (green) was viewed by confocal microscopy in optical sections midway through the cell. Cell nuclei (blue) were labeled with 4′,6-diamidino-2-phenyl indole dihydrochloride (DAPI). Briefly, the monolayer cultures were rinsed and fixed with 4% paraformaldehyde in HBSS; DAPI (1 μg/ml) was added immediately after cell fixation for 15 min, and the cells were rinsed three times and finally mounted on glass slides using ProLong antifade mounting medium (Molecular Probes, Eugene, OR). Confocal images were acquired with a laser-scanning confocal microscopy (Zeiss LSM 510; Thornwood, NY) with a 488-nm excitation laser line to detect Alexa 488 (BP505–550 nm emission), and nonconfocal DAPI images were acquired using Hg lamp excitation with the ultraviolet filter set (BP385–470 nm emission) respectively. Optical sections had a thickness of less than 1 μm (pinhole set to achieve 1 Airy unit). Quantitative analysis of images (average whole cell fluorescence intensity) in the acquired confocal images (n = 6/treatment group) was determined using Zeiss LSM 510 META software. The average background fluorescence detected from areas where no cells were present in each experimental condition was subtracted from the total fluorescence to yield specific cellular fluorescence intensity.

Transendothelial ^{125}I-Albumin Transport. Rat lung microvascular endothelial cells were grown on clear microporous polyester Transwell membranes (12 wells, 1 cm^2 growth area, 0.4 μm pore size; Corning Costar, Cambridge, MA). The membrane inserts (inner well) were filled with a total of 0.5 ml HBSS containing ^{125}I-albumin (1 $\times$ 10^6 cpm) in the presence of unlabeled albumin (0.1 or 100 mg/ml). The lower well was filled with 1.5 ml HBSS of the same osmolarity as the inner well. Thus, fluid levels and osmotic pressure in the "upper" and "lower" wells were equalized to eliminate

hydrostatic and osmotic pressure differences across the monolayer. Aliquots of 500 μl were sampled from the lower chamber after exposure to air or 1.0 and 2.0 MAC isoflurane or sevoflurane at 37°C for 30 min, and then γ radioactivity of the samples was measured. Transendothelial ^{125}I-albumin permeability was calculated from the flux of radiolabeled albumin across the cell monolayer, as described previously.[23,24] Albumin permeability is expressed in units of $\mu l \cdot min^{-1} \cdot cm^{-2}$.

The role of caveolae in effects of 2.0 MAC isoflurane and sevoflurane on transendothelial albumin transport was also evaluated in RLMVECs with methyl-β-cyclodextrin using the same basic protocol. Before the administration of volatile anesthetic, endothelial cells were pretreated for 15 min with 2.0 mM methyl-β-cyclodextrin, followed by two washes.

Western Blot Analysis. Phosphorylation of caveolin-1 and *Src* kinase in RLMVECs was determined by Western blot analysis. Confluent RLMVEC monolayers on six-well plates were treated with 2.0 MAC isoflurane or sevoflurane at 37°C for 30 min. Cell lysates were subsequently prepared as described,[25,26] and the protein concentration in the lysate was assayed. Equal amounts of protein (20 μg) were loaded on 12% acrylamide gels, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Nonspecific binding was blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature. The membranes were washed three times with TBS-T solution (0.05% Tween 20 in TBS) and incubated with tubulin monoclonal antibody (1:5,000) in 5% nonfat dry milk, pY14-caveolin-1 (1:1,000), or pY416-*Src* polyclonal antibodies (1:1,000) in TBS-T solution. Incubation was carried out overnight; the membranes were washed three times for 5 min each and then incubated for 60 min with goat anti-rabbit (polyclonal) or anti-mouse (monoclonal) immunoglobulin G conjugated to horseradish peroxidase (1:5,000). Membranes were washed three times for 5 min each, and the protein bands were detected using the ECL reagent (Pierce, Rockford, IL). Molecular mass of the proteins was determined using known marker proteins. Relative intensity of tubulin, phosphorylated caveolin-1, and *Src* was measured using Scion Image (National Institutes of Health, Bethesda, MD).

Drugs and Reagents

The chemicals and reagents used were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. Horseradish peroxidase–conjugated goat anti-rabbit and anti-mouse immunoglobulin G were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). pY14-caveolin-1 and pY416-*Src* polyclonal antibody were from Chemicon (Temecula, CA) and Cell Signaling Technology, Inc. (Beverly, MA), respectively. Goat anti-mouse and anti-rabbit immunoglobulin G, Alexa 488-albumin, and DAPI were purchased from Molecular Probes, Inc. (Eugene, OR). HBSS containing $NaHCO_3$ (4.2 mM) and HEPES (10 mM) was adjusted to pH 7.4. Bovine serum albumin (fraction V, 99% pure, endotoxin free, cold alcohol precipitated) and methyl-β-cyclodextrin were dissolved in HBSS. Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY) and Hyclone (Logan, UT), respectively. ^{125}I-labeled albumin was purchased from PerkinElmer, Inc. (Wellesley, MA). Trichloroacetic acid precipitation analysis was used to confirm the purity[27]; free ^{125}I was removed from ^{125}I-labeled albumin with a Sephadex G25 column such that the contaminant free ^{125}I in the tracer used contributed less than 0.3% of the total counts.

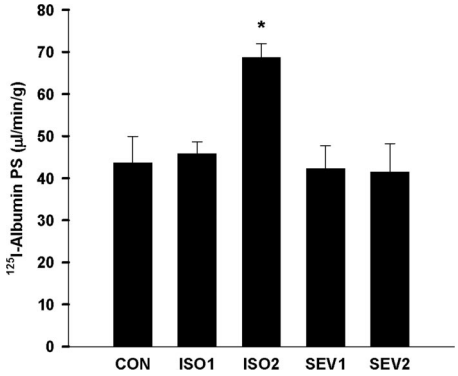

Fig. 1. Effects of 1 and 2 minimum alveolar concentration isoflurane (ISO1 and ISO2) and sevoflurane (SEV1 and SEV2) on ^{125}I-albumin permeability–surface area (PS) product in the isolated rat lung. Values are mean ± SD of at least six lungs. * $P <$ 0.05 *versus* control group (CON).

Statistical Analysis

One-way analysis of variance and the Student-Newman-Keuls test for *post hoc* comparisons were used to determine differences between control and experimental groups. The Student *t* test was performed for paired samples. Data were expressed as mean ± SD. Differences were considered significant at $P < 0.05$.

Results

Effect of Volatile Anesthetics on Endothelial Albumin and Liquid Permeability in the Isolated Rat Lung

Initial baseline values for pulmonary artery pressure were similar for the experimental groups. There was no change in pulmonary artery pressure (10–15 cm H_2O) among different groups after administration of 1.0 or 2.0 MAC isoflurane or sevoflurane for 30 min.

Compared with the control group, isoflurane at 2.0 MAC increased ^{125}I-albumin PS product (a measure of protein permeability) in the isolated, perfused rat lung by 48% (fig. 1), whereas 1.0 MAC isoflurane and sevoflurane and 2.0 MAC sevoflurane had no effect on ^{125}I-albumin PS product. $K_{f,c}$ (a measure of liquid permeabil-

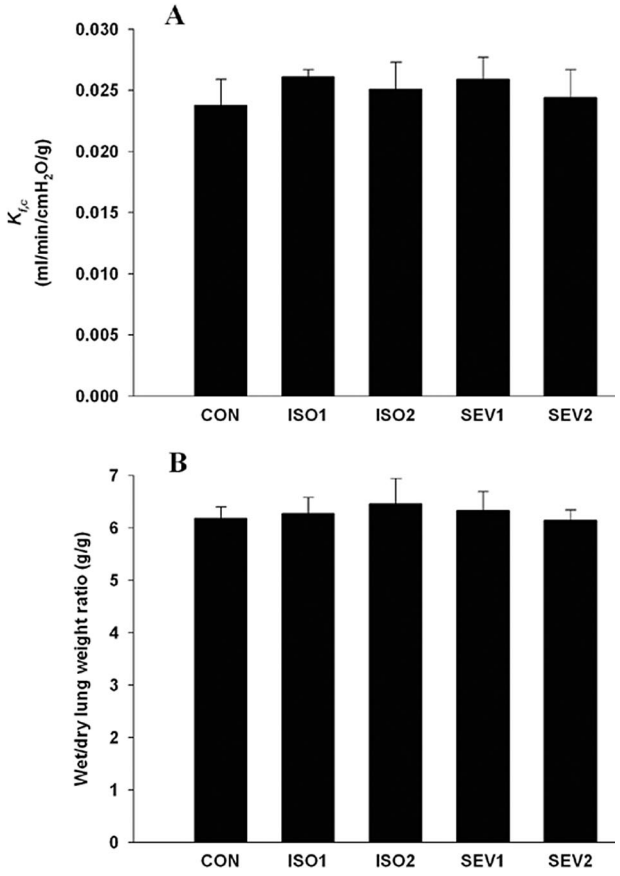

Fig. 2. Effects of 1 and 2 minimum alveolar concentration isoflurane (ISO1 and ISO2) and sevoflurane (SEV1 and SEV2) on capillary filtration coefficient ($K_{f,c}$) (*A*) and wet/dry lung weight ratio (*B*) in the isolated rat lung. Values are mean ± SD of at least six lungs. CON = control.

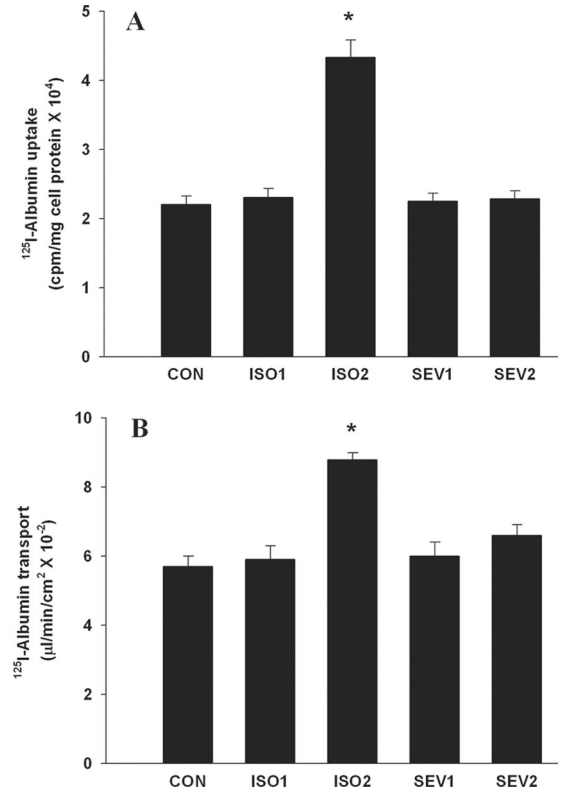

Fig. 3. Effects of 1 and 2 minimum alveolar concentration isoflurane (ISO1 and ISO2) and sevoflurane (SEV1 and SEV2) on uptake (*A*) and transendothelial transport (*B*) of ^{125}I-albumin in rat lung microvascular endothelial cell monolayers. Values are mean ± SD of at least four observations. * $P < 0.05$ *versus* control group (CON).

ity) at baseline was similar in all groups. Neither 1.0 and 2.0 MAC isoflurane nor sevoflurane altered the $K_{f,c}$ (fig. 2A). In addition, the wet/dry lung weight ratio (lung fluid content) was not significantly different in the various groups (fig. 2B).

Effect of Volatile Anesthetics on Albumin Uptake and Transport in RLMVECs

In wild-type RLMVECs, isoflurane at 2.0 MAC more than doubled the uptake of ^{125}I-albumin (4.33 ± 0.25 *vs.* 2.16 ± 0.12 cpm/mg cell protein × 10^4; fig. 3A) and caused an approximately 54% increase in transendothelial transport of ^{125}I-albumin (8.78 ± 0.22 *vs.* 5.70 ± 0.21 ml · min^{-1} · cm^{-2} × 10^{-2}; fig. 3B). Isoflurane and sevoflurane at 1.0 MAC and sevoflurane at 2.0 MAC did not alter ^{125}I-albumin uptake and transport in cultured cells. Consistently, confocal images showed that 2.0 MAC isoflurane caused a fourfold increase in fluorescent albumin uptake, whereas 2.0 MAC sevoflurane had no effect on fluorescent albumin uptake in RLMVECs (fig. 4). The mean specific fluorescence intensity under control conditions was 37 ± 2 fluorescence units/pixel, which increased to 153 ± 27 in cells treated with 2 MAC isoflurane and to 47 ± 4 in cells treated with sevoflurane. Furthermore, the effect of isoflurane on fluorescent albumin uptake was abolished in cells expressing the phosphorylation defective caveolin-1 mutant, Y14F-Cav-1 (fig. 4); the specific fluorescence intensity of internalized albumin was less than that observed under control conditions (19 ± 6 fluorescence units/pixel; $P <$ 0.05 *vs.* control).

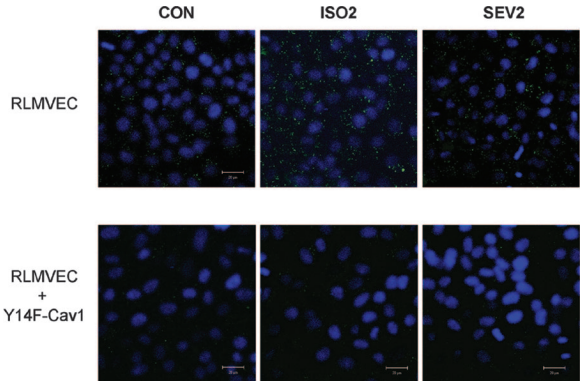

Fig. 4. Confocal images showing Alexa 488-albumin (*green*) uptake and DAPI staining of the nucleus (*blue*) in rat lung microvascular endothelial cells (RLMVECs) (*top*) or RLMVECs expressing Y14F-Cav-1 (*bottom*) after exposure to 2.0 minimum alveolar concentration isoflurane (ISO2) and sevoflurane (SEV2). CON = control.

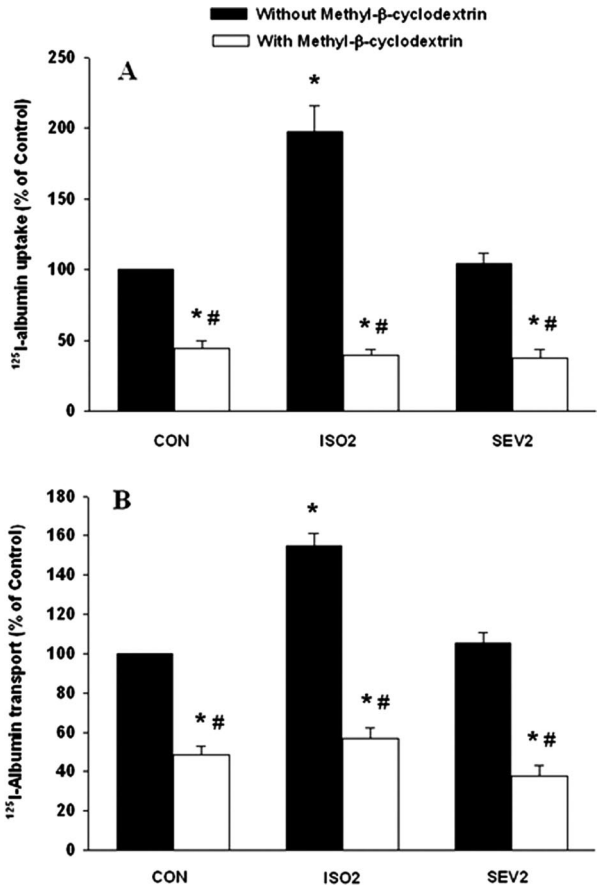

Fig. 5. Effects of pretreatment of endothelial cells with 2 mM methyl-β-cyclodextrin on uptake (*A*) and transendothelial transport (*B*) of ^{125}I-albumin in the absence and presence of 2.0 minimum alveolar concentration isoflurane (ISO2) or sevoflurane (SEV2). Values are mean ± SD of at least four observations. * *P* < 0.05 *versus* control group (CON). # *P* < 0.05 *versus* corresponding groups.

To test whether the isoflurane-induced increase in albumin uptake and transendothelial transport are mediated by caveolae, we pretreated RLMVECs for 15 min with methyl-β-cyclodextrin, a membrane cholesterol–depleting agent that thereby flattens caveolae, and then observed the effects of isoflurane on ^{125}I-albumin uptake and transport. Our results indicated that pretreatment of RLMVECs with methyl-β-cyclodextrin reduced uptake and transendothelial transport of albumin to less than 50% of control levels in the absence or presence of 2.0 MAC isoflurane or sevoflurane (fig. 5).

To address further the role of caveolin-1 in the isoflurane-induced increase in albumin permeability, we compared the effects of isoflurane on ^{125}I-albumin uptake in wild-type and Y14F-caveolin-1 mutant RLMVECs. Isoflurane caused an approximately 100% increase in ^{125}I-albumin uptake in wild-type RLMVECs, and interestingly, this effect was abolished in Y14F-caveolin-1 mutant expressing cells (fig. 6). This key observation demonstrates that the isoflurane-induced increase in albumin uptake is dependent on and requires caveolin-1 phosphorylation.

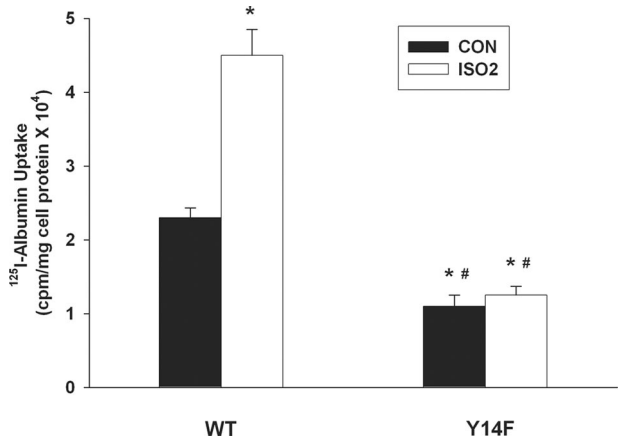

Fig. 6. Effects of 2.0 minimum alveolar concentration isoflurane (ISO2) on uptake of ^{125}I-albumin in wild-type (WT) and Y14F-caveolin-1 mutant expressing cells (Y14F). Values are mean ± SD of at least four observations. * *P* < 0.05 *versus* WT control group (CON). # *P* < 0.05 *versus* corresponding groups.

Effect of Volatile Anesthetics on Phosphorylation of Caveolin-1 and Src Family Kinase

Figure 7 shows that in comparison with the control group, 2.0 MAC isoflurane caused a twofold increase in phosphorylation of both caveolin-1 and *Src* family kinase in wild-type RLMVECs (fig. 7A) but had no effect in Y14F-caveolin-1 mutant expressing cells (fig. 7B). Equal protein loading per lane was confirmed by reprobing the blots with antitubulin antibody, which showed similar tubulin protein bands in each groups. Increase in phospho-*Src* (PY-416) immunoblot indicates *Src* activation, and increased phosphorylation of caveolin-1, which we previously showed is mediated by activated *Src* kinase,[26,28] is indicative and required for caveolae-mediated endocytosis and transcytosis.[19] Sevoflurane at 2.0 MAC did not alter phosphorylation of caveolin-1 and *Src* family kinase (fig. 7A).

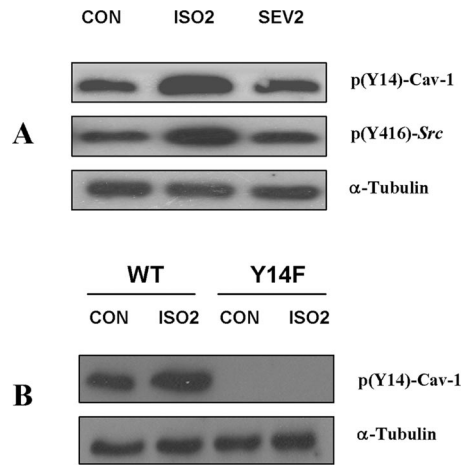

Fig. 7. Effects of 2.0 minimum alveolar concentration isoflurane (ISO2) and sevoflurane (SEV2) on phosphorylation of caveolin-1 (p(Y14)-Cav-1) and *Src* family kinase (p(Y416)-*Src*) (*A*) and effects of ISO2 on p(Y14)-Cav-1 in wild-type (WT) and Y14F-caveolin-1 mutant expressing endothelial cells (Y14F) (*B*). CON = control.

Discussion

At identical minimum alveolar concentrations (corresponding to nearly equal aqueous molar concentrations at 37°C), isoflurane (0.6 mM), but not sevoflurane (0.7 mM), activated albumin transcytosis in endothelial cells, indicating that isoflurane has properties not shared by sevoflurane. The physiologic activator of albumin transcytosis is the albumin molecule, which engages a specific receptor molecule (gp60) on the endothelial cell surface.[25–29] Upon binding to gp60, albumin activates *Src*, which enhances the phosphorylation of caveolin-1.[19,26–29] Hence, *Src* activation is a critical regulatory event required for the initiation of albumin transcytosis through endothelium.[19,26–29] We now show that the presence of isoflurane further promotes *Src* activation and caveolin-1 phosphorylation, thereby resulting in augmented albumin transcytosis as observed.

The effect of volatile anesthetics on albumin transport across the vessel wall has not been previously investigated. The current findings show that isoflurane increases transendothelial transport of albumin (albumin PS product) in an *ex vivo* lung preparation. Because no change in pulmonary artery pressure, venous pressure, or perfusion rate occurred during the experiments conducted, the increase in albumin transport cannot be attributed to increased capillary hydrostatic pressure and the resultant convective flux of albumin. Albumin transport across the endothelial barrier occurs by means of two distinct pathways, *i.e.*, a transcellular pathway (caveolae-mediated) and a paracellular pathway (diffusion).[2,3] In the current study, however, isoflurane increased albumin PS product without causing changes in $K_{f,c}$ or wet/dry lung weight ratio, suggesting that isoflurane did not affect the integrity of interendothelial cell junctions. These results indicate that albumin transport stimulated by isoflurane used a nonhydraulic pathway to cross the vascular endothelial barrier.[12,29]

The cellular barrier to proteins in lung microvessels consists of an endothelial cell layer with highly restrictive interendothelial junctions. To identify the albumin-transporting mechanism, we designed studies on rat pulmonary endothelial cell monolayers, which form a similarly restrictive barrier *in vitro*.[21] We first showed that isoflurane (but not sevoflurane) increased monolayer permeability, using the same anesthetic concentration effective in stimulating albumin uptake in the *ex vivo* lung preparation. These experiments showed that endothelial cells indeed represent a significant site of action of isoflurane. The *in vitro* approach was advantageous by allowing analysis of cellular internalization of iodinated or fluorescent albumin tracers. Analysis of cell lysates for iodinated albumin indicated that isoflurane (but not sevoflurane) activated the cellular uptake of albumin, and imaging studies confirmed that isoflurane stimulated fluorescent tracer uptake (fluorescent forms of albumin or cholera toxin) into punctate intracellular structures that we previously demonstrated were positive for caveolin-1.[21,29] Our previous confocal imaging studies showed that Alexa 488-albumin colocalizes in endocytic vesicles with Alexa 594-cholera toxin subunit B, a specific caveola marker in endothelial cells.[21,29] These observations indicate that isoflurane activated caveolae-mediated uptake of tracer protein molecules. The additional observation that isoflurane stimulated transendothelial transfer of tracer molecules across the entire endothelial monolayer further indicated that caveolae participated in a transendothelial transport process, *i.e.*, transcytosis. Blocking of the transendothelial transport process with the cholesterol-depleting agent cyclodextrin confirmed this expectation. Cyclodextrin was used for its ability to deplete endothelial caveolae.[4,21] Because cyclodextrin completely eliminated the isoflurane stimulated transport process, we are able to rule out the paracellular pathway as a significant mode of transendothelial transport in the case of isoflurane.

It is known that albumin binding to the 60-kd glycoprotein (gp60) on the endothelial cell surface induces clustering of gp60 and its physical interaction with caveolin-1.[29] *Src* tyrosine kinase is bound to caveolin-1 *via* its scaffolding domain,[30] palmitoylated C-terminal cysteine residue, and N-terminal phosphorylated tyrosine residue,[19] and the kinase is activated upon albumin binding to gp60.[27] Activated *Src*, in turn, phosphorylates caveolin-1, gp60, and dynamin-2 to initiate plasmalemmal vesicle fission and transendothelial vesicular transport of albumin.[26–28] The current findings provide additional support for this hypothesis by indicating that isoflurane increases albumin uptake and transport in endothelial cells and that these effects are associated with enhanced *Src* activation and phosphorylation of caveolin-1. We also observed that the increase in uptake of albumin (induced by isoflurane) was abolished in endothelial cells expressing the phosphorylation-defective Y14F-caveolin-1 mutant. These data show that caveolin-1 phosphorylation is a crucial event in isoflurane-induced increase in albumin uptake.

Our data showed that isoflurane increased the phosphorylation of *Src* at tyrosine 416, which is required for activation of *Src*.[28] Moreover, our previous studies demonstrated that the phosphorylation of caveolin-1 and albumin uptake were abolished in RLMVECs transfected with dominant-negative *Src*.[26,28] Our current results dovetail with our previous observations by indicating that *Src* activation (mediated by isoflurane) stimulates albumin uptake in endothelial cells. This conclusion is consistent with the pharmacologic evidence for *Src* activation by isoflurane in the rat heart, using the *Src* inhibitor 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolol[3,4-day]pyrimidine (PP1).[31]

The mechanism by which isoflurane activates Src kinase remains to be clarified. One possibility is a direct

activation of *Src* by isoflurane. It is known that the potency or efficacy of volatile anesthetics correlates with lipid solubility, protein perturbation, or both. Because of its lipid solubility, anesthetic molecules can interact at the molecular level with lipids and proteins.[32] Therefore, we speculate that isoflurane may accumulate in caveolae and directly activate *Src*, which is normally present bound to caveolin-1.[26] Other potential mechanisms for *Src* activation include (1) displacement of *Src* from caveolin-1, which is thought to negatively regulate *Src* activity[33]; (2) activation of an upstream stimulatory kinase[34]; or (3) inhibition of C-terminal Src kinase, which negatively regulates *Src* activity by phosphorylating *Src* on tyrosine 527.[35]

Noteworthy was that sevoflurane, in contrast to isoflurane, did not alter albumin uptake and transport in RLMVECs. Consistent with these functional observations, we showed that sevoflurane lacks an ability to activate *Src* or induce caveolin-1 phosphorylation in the RLMVECs. These observations reinforce the view that isoflurane acts by stimulating *Src* activity and caveolin-1 phosphorylation.

There are several explanations for the absence of pulmonary edema (in the isolated rat lung preparation) despite increased transendothelial albumin permeability. Increased transport of albumin from blood into the interstitium may be compartmentalized in an "excluded volume" within the interstitial space, because of its large molecular size and/or charge density.[36] This interstitial albumin exclusion is thought to serve as an important buffering mechanism to prevent development of pulmonary edema.[12,36] Another possibility is that the increased interstitial pressure due to a slight increase in fluid filtration caused by isoflurane (secondary to extravasation of albumin) buffers or even nullifies the Starling pressure gradient that causes microvascular fluid filtration.[37] The normal lung has a low interstitial compliance provided by the inflexible structure of the subendothelial matrix, such that a small increase in interstitial volume provokes a marked increase in tissue pressure.[37–39] In the Starling equation, increased tissue pressure favors fluid reabsorption by capillaries and limits fluid filtration. Low compliance also represents an important "tissue safety factor" to counteract further progression of pulmonary edema.[36–40] Another possible explanation is that isoflurane may also affect albumin transport in the reverse direction, *i.e.*, from the interstitium to the lumen (abluminal to luminal transport).[41] Thus, isoflurane-induced increase in extravascular albumin clearance may limit the accumulation of interstitial albumin and an increase in oncotic pressure, and therefore, no increase in lung weight as a consequence of an increase in extravascular osmolarity would be observed.

Our findings are consistent with studies that evaluated the effect of volatile anesthetics on alveolar epithelial and capillary permeability in the isolated perfused rabbit lung[8,10] and in the rat[9] and human.[11] These studies indicated that isoflurane increased pulmonary capillary permeability but had no effect on tissue edema in the normal lung. Recently, other studies have been performed to test the effect of isoflurane on tissue edema during pathologic conditions and showed that isoflurane enhanced the development of neurogenic pulmonary edema in rats[42] and caused increased interstitial fluid accumulation in the crystalloid volume-loaded sheep.[43] Although neurohumoral regulatory mechanisms (*e.g.*, vascular endothelial growth factor, neuropeptide Y, atrial natriuretic peptide) have been suggested in these *in vivo* studies, local and direct effects of isoflurane on pulmonary endothelial cells (*i.e.*, endothelial albumin permeability) could not be ruled out. Therefore, our study, when combined with these *in vivo* results, suggests the possibility that isoflurane may decrease the threshold for alveolar edema in patients at high risk for pulmonary edema.

In summary, the current study demonstrated that isoflurane, but not sevoflurane, increases pulmonary endothelial albumin permeability and both albumin internalization and transendothelial albumin transport in cultured endothelial cells. Isoflurane increased *Src* activation and caveolin-1 phosphorylation, which we previously showed were critical events regulating caveolae-mediated endocytosis and transcytosis.[19,26–29] The isoflurane-induced increase in albumin permeability was not associated with net fluid movement in the normal lungs, probably because several safety factors operate to prevent edema. Conditions such as sepsis and inflammation, known to reduce pulmonary safety factors, may complicate use of anesthetic agents with permeability increasing properties. Isoflurane-induced *Src* activation, resulting in caveolin-1 phosphorylation, may be an underlying molecular mechanism critical to the regulation of vascular protein permeability.

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