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The Immunomodulatory Effect of Sevoflurane in Endotoxin-Injured Alveolar Epithelial Cells

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BACKGROUND: Endotoxin-induced lung injury is a useful experimental system for the characterization of immunopathologic mechanisms in acute lung injury. Although alveolar epithelial cells (AEC) are directly exposed to volatile anesthetics, there is limited information about the effect of anesthetics on these cells. In this study we investigated the effect of pretreatment with the inhaled anesthetic sevoflurane on lipopolysaccharide (LPS)-injured AEC.

METHODS: AEC were incubated with 1.1 vol % sevoflurane for 0.5 h, followed by LPS stimulation for 5 h. Expression of monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), macrophage inflammatory protein-2 (MIP-2), cytokine-induced neutrophil chemoattractant-1 (CINC-1), and intercellular adhesion molecule-1 (ICAM-1) was analyzed. In addition, functional tests were performed through chemotaxis and adherence assays to underline the biological relevance of the findings.

RESULTS: Exposure of AEC to sevoflurane resulted in a 50% downregulation of MCP-1 protein in the sevoflurane-LPS group when compared with non-sevoflurane-LPS cells ($P < 0.05$). MIP-1β concentration in LPS-stimulated cells decreased by 32% with sevoflurane ($P < 0.05$), MIP-2 by 29% ($P < 0.05$), and CINC-1 by 20% ($P < 0.05$). ICAM-1 protein expression was attenuated by 36% ($P < 0.05$). This inhibition caused substantial changes in the inflammatory response of neutrophils. 33% less chemotactic activity was seen in sevoflurane-treated LPS cells ($P < 0.001$) as well as 47% decreased adhesion of neutrophils to AEC ($P < 0.001$).

CONCLUSIONS: This study shows that sevoflurane alters the LPS-induced inflammatory response, not only with respect to the expression pattern of inflammatory mediators, but also regarding the biological consequences with less accumulation of effector cells such as neutrophils.

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Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are characterized by a spectrum of increasingly severe lung injury in which widespread damage to cells and structures of the alveolar capillary membrane occurs within hours to days after a predisposing insult (1). ALI/ARDS is a major cause of acute respiratory failure with frequent morbidity and mortality in critically ill patients (2). The incidence of ARDS may even increase significantly in the future because of increasing frequency of predisposing conditions that precipitate ALI/ARDS, such as sepsis (3). Although mortality in patients with ALI/ARDS may have declined over the last 15 yr, it remains high (30%–40%) (4).

Lipopolysaccharides (LPS) are bacterial endotoxins which can lead to inflammatory reactions with pathological events resembling ALI/ARDS. LPS upregulates the expression and production of a number of chemokines such as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), macrophage inflammatory protein-2 (MIP-2), cytokine-induced neutrophil chemoattractant-1 (CINC-1), as well as adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) *in vitro* and *in vivo* (5–8). In the respiratory compartment, alveolar epithelial cells (AEC) are involved in the inflammatory response upon stimulation with LPS (9–11). Through the expression and production of these inflammatory mediators, not only the vascular but also the airway epithelium is likely to play an important role in the initiation and exacerbation of an inflammatory response within the lungs.

In the past, volatile anesthetics have been shown to attenuate cardiac mechanical dysfunction and limit ultrastructural abnormalities on reperfusion after ischemia in the myocyte (12). Preconditioning by anesthetics has become a main topic of cardiac anesthetic

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research (13). Although volatile anesthetics are delivered through the lung, there is only limited information about their effect on different lung cells such as AEC. Therefore, we hypothesized that the inflammatory response of AEC to LPS-induced lung injury may also be altered by pretreatment with volatile anesthetics.

METHODS

Alveolar Epithelial Cells (AEC)

L2 cells (CCL-149) were purchased from American Type Culture Collection (Rockville, MD). The alveolar epithelial L2 cell line was derived through clonal isolation after primary plating of a cell population from enzymatically dispersed adult rat lung. AEC were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen AG, Basel, Switzerland), which was supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% HEPES buffer. Using 35 × 10 mm plates (Corning, Corning, NY) AEC were grown until confluence.

Sevoflurane Exposure and Stimulation with LPS

Medium of confluent monolayers of AEC was changed to DMEM/1% FBS and placed in a preheated (37°C) 3.5 L air-tight chamber (Oxoid, Basel, Switzerland).

In a first approach AEC were exposed to 0.5, 1.1, 2.2, 3.3 vol % sevoflurane (Sevorane®, Abbott, Switzerland) for 0.5, 2, 4, and 12 h. A mixture of 5% CO_2 and 95% air was directed through a Sevotec 5 Vaporizer (Abbott, Switzerland), placed at the entrance of the chamber (for controls only CO_2/air mixture). These sevoflurane concentrations were chosen according to the one minimum alveolar concentration (MAC) value of 2.2 vol % for this anesthetic in young humans (0.5, 1.1, 2.2, and 3.3 vol % representing 0.25, 0.5, 1, and 1.5 MAC). A 5330 Agent Monitor (Datex-Ohmeda, Duisburg, Germany) determined the concentration of the volatile anesthetic exiting the chamber. The chamber was sealed upon reaching the desired concentration and incubated at 37°C. The chamber atmosphere was kept saturated continuously with H_2O. After exposure of AEC to the volatile anesthetic, cells were washed twice with warm phosphate-buffered saline (PBS) and incubated with LPS from *Escherichia coli* serotype 055:B5 (LPS; 20 μg/mL; Sigma-Aldrich, Buchs, Switzerland) (or PBS as a control) for 1, 3, 5, 7, 12, 18, 24 h in a normal incubator with 5% CO_2 and 95% air at 37°C.

Concentrations of the volatile anesthetic were controlled. There was no variation between the concentration measured before sealing the chamber and the end of the experiment.

Viability of cells was determined by the trypan blue dye exclusion assay. In addition, results were confirmed with a cytotoxicity assay (determination of lactate dehydrogenase) (Promega, Madison, WI).

RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total cellular RNA was isolated from treated AEC. Following manufacturer's instructions, the cells were lysed by adding monophasic solution of phenol and guanidine isothiocyanate (TRIzol® Reagent, Invitrogen AG, Basel, Switzerland). RNA was precipitated with chloroform and isopropanol, washed with 75% ethanol, and finally dissolved in RNAase-free water. Total amounts of cellular RNA were determined by absorbance at 260 nm.

Random hexanucleotide primers and murine leukemia virus reverse transcriptase were used for cDNA synthesis. Reverse transcription was performed with 0.8 μg RNA at 20°C for 5 min, 42°C for 30 min and 99°C for 5 min. Specific primers located on separate exons were designed to asses gene expression of MCP-1, MIP-2, CINC-1, and ICAM-1 in AEC (summarized in Appendix 1 available at *www.anesthesia-analgesia.org*).

RT-PCR reaction products were resolved on 1.5% agarose gels (Invitrogen AG) and stained with ethidium bromide. Polymerase chain reaction was also performed with 18S primers to ensure equal loading.

Enzyme-Linked Immunosorbent Assay for MCP-1, MIP-2, and CINC-1

To measure MCP-1, MIP-2, and CINC-1 protein production in the supernatant of AEC, ELISA was performed according to the manufacturer's recommendations, using OptEIA™ rat MCP-1 ELISA (Pharmingen, San Diego) and rat MIP-2 and CINC-1 ELISA (R&D Systems, Abingdon, UK), respectively. Optical density was determined at 492 nm and the results were given in pg/mL.

Western Blot Analysis for MIP-1β

Characterization of proteins was performed using a SDS-polyacrylamide gel. Twenty-five microliters of collected supernatants of AEC were electrophoresed and after separation, proteins were transblotted to a nitrocellulose membrane for 2 h at 200 mA. After washing with PBS, the membrane was blocked with PBS/4% low fat milk/0.1% Tween 20 for 1 h at room temperature, followed by overnight incubation with a polyclonal rabbit anti-rat MIP-1β antibody (Biosource Int. Camarilla, CA) in blocking buffer at 4°C. All washing steps were performed three times with PBS/0.1% Tween 20. A secondary horseradish peroxidase-labeled anti-rabbit IgG (1:5000) (Sigma-Aldrich) in blocking buffer was added for 1 h at room temperature. Signals were detected by enhanced chemiluminescence.

Cell-Based ELISA for ICAM-1

AEC were added to 96-well plates and grown to confluence at 37°C in 5% CO_2. Cells were exposed to sevoflurane and stimulated with LPS, as described earlier. After washing once with PBS, cells were fixed

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with PBS/3% paraformaldehyde for 15 min at room temperature (14). After each incubation step, the cells were washed three times with PBS. Incubation overnight at 4°C with the primary antibody was performed with monoclonal mouse anti-rat ICAM-1 antibody (1A29; Serotec, Oxford, UK) diluted in PBS/5% FBS to a concentration of 0.5 μg/mL. After a washing step, the cells were incubated with the secondary goat anti-mouse IgG, conjugated with horseradish peroxidase (1:2500 in PBS/5% FBS; 1 h at room temperature). The washing procedure was repeated and 200 μL of *o*-phenylenediamine dihydrochloride solution (Sigma-Aldrich) was added to each well and plates were incubated for min at room temperature in the dark. Finally, the reaction was stopped with 3 M H_2SO_4 and the optical density was determined at 492 nm. The optical density values were interpreted as up or downregulation of ICAM-1 when compared with control groups.

Preparation of Neutrophils (Polymorphonuclear Cells, PMN)

Human neutrophils were isolated by gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Dubendorf, Switzerland) followed by 1% dextran sedimentation for 1 h to separate neutrophils from erythrocytes (9). After centrifugation of the supernatant, the contaminating erythrocytes were lysed with distilled water followed by the addition of 2.7% NaCl to stop hypotonic lysis. The remaining neutrophils were resuspended in DMEM/1% FBS. Neutrophils were labeled at a total concentration of 5×10^6 PMN/mL and a concentration of 5 μM of the fluorescent indicator calcein acetoxymethyl ester (Calcein AM, Calbiochem® Biochemicals, Juro supply, Switzerland) for 30 min at 37°C. PMN were subsequently washed twice with PBS and resuspended in DMEM/1% FBS at the desired concentration.

Chemotaxis Assay

1×10^5 calcein-AM-labeled neutrophils were given into the upper chamber of MultiScreen-MIC filter plates (Millipore, France). Receiver plates were loaded with 150 μL of supernatant of previously treated cells as described earlier (5 h LPS stimulation). The chemotactic substance *N*-formylmethionyl-leucyl-phenylalanine 0.1 μM, (Sigma-Aldrich) was taken as positive control, while DMEM/1% FBS was used to determine basal migration. After 2 h incubation at 37°C, the filter plate was taken out and migrated cells were lysed with 1.0% Triton-X (Sigma-Aldrich). Fluorescence was measured by using an excitation filter at 485 nm and an emission filter at 535 nm. The ratio between migration to supernatant of stimulated AEC and basal migration was used as indication of PMN chemotaxis.

Assay of Neutrophil Adherence to AEC

AEC were added to 6-well plates and grown to confluence at 37°C in 5% CO_2. Cells were exposed to sevoflurane and stimulated with LPS as described earlier (5 h LPS stimulation). AEC were washed with DMEM after stimulation and 5×10^5 calcein-AM-labeled PMN were added to each well for 30 min at 37°C. Nonadherent PMN were removed by carefully washing the cells twice with DMEM. DMEM 100 μL was added to each well before fluorescent measurement and then fluorescence was measured using an excitation filter at 485 nm and an emission filter at 535 nm. The amount of adherent PMN was calculated by using a standard row.

For ICAM-1-blocking studies on AEC after stimulation, cells were preincubated with anti-ICAM-1 (1A29) or control antibody (MOPC-21, Serotec, Oxford, UK) at a concentration of 10 μg/mL at 37°C for 20 min (14). At the same time, neutrophils were preincubated with anti-FcγRIII (CD16) and anti-FcγRII (CD32) antibodies (Becton Dickinson, Switzerland) at a concentration of 10 μg/mL for each antibody in DMEM at 37°C for 15 min. AEC and neutrophils were washed once with DMEM and incubated together as described earlier.

Statistical Analysis

In all experiments, statistical significance was evaluated using the paired Student's *t*-test at a 5% level. Each experimental setup was performed five times. Values are expressed as mean ± SEM. To calculate percent protection, all values after LPS stimulation were adjusted by subtraction of negative control values. Non-sevoflurane-LPS groups were then compared with sevoflurane-LPS groups.

RESULTS

All results presented from RT-PCR were analyzed by densitometry and presented in a quantitative manner with regard to the fact that RT-PCR is only a semiquantitative method. Original blots are available at *www.anesthesia-analgesia.org*.

Evaluation of Optimal Pretreatment of AEC with Sevoflurane

In a first approach, different concentrations of the volatile anesthetic were evaluated to determine the optimal effect of pretreatment of AEC with sevoflurane. AEC were exposed to 0.5, 1.1, 2.2, and 3.3 vol % sevoflurane for 0.5 h, followed by LPS stimulation for 3 h. To evaluate the effect of time regarding pretreatment of AEC with 1.1 vol % sevoflurane, cells were exposed to the anesthetic for various time periods (0.5, 2, 4, and 12 h), followed by stimulation with LPS for 3 h (summarized in Appendix 2 available at *www.anesthesia-analgesia.org*).

For all these experiments, decreased expression of inflammatory mediators due to increased rate of cell necrosis could be excluded with trypan blue staining and lactate dehydrogenase assays.

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These preliminary experiments showed that a sevoflurane exposure time of 0.5 h with a concentration of 1.1 vol % induced the most pronounced suppression regarding MCP-1 expression. Therefore, the following experiments were performed under these conditions.

Effect of Various LPS Exposure Times on MCP-1 Expression in AEC After Sevoflurane Pretreatment

To evaluate the effect of sevoflurane at various time points after LPS stimulation, cells were exposed to sevoflurane for 0.5 h with a concentration of 1.1 vol %, followed by a stimulation with LPS 20 μg/mL or PBS as a control. AEC were stimulated with LPS for 1, 3, 5, 7, 12, 18, and 24 h. The most pronounced increase of mRNA for MCP-1 was seen after 7–12 h of LPS exposure with more than 200% enhanced expression ($P < 0.05$) versus the control group. After sevoflurane pretreatment, a statistically significant downregulation of mRNA for MCP-1 was found at 1, 3, and 5 h LPS exposure (63%, 139%, 41%, $P < 0.05$) and after 12 and 24 h (both 37%, $P < 0.05$). No difference was seen after 7 and 18 h (Fig. 1A). This downregulation of MCP-1 mRNA showed a biphasic pattern. All densitometries were compared with 18S (figures not shown).

MCP-1 protein concentration in supernatants of non-sevoflurane-LPS exposed AEC was upregulated from 0.4 ± 0.0 ng/mL in the non-sevoflurane-PBS group to 147.8 ± 6.5 ng/mL at 24 h in the non-sevoflurane-LPS group ($P < 0.001$). Exposing cells to sevoflurane before LPS-stimulation showed the following downregulation when compared with non-sevoflurane-LPS cells: at 1 h, MCP-1 was downregulated by 9% ($P < 0.05$), at 3 h by 16% ($P < 0.05$), at 5 h by 50% ($P < 0.05$), at 12 h by 10% ($P < 0.01$), and at 24 h by 21% ($P < 0.05$). There was no statistically significant difference after 7 and 18 h of LPS stimulation (Fig. 1B).

Effect of Various LPS Exposure Times on MIP-1β, MIP-2, CINC-1, and ICAM-1 Expression in AEC After Sevoflurane Pretreatment

Although several primers had been tested for mRNA for MIP-1β, results were not conclusive. Therefore, we focused on the protein only, as this also represents the major component for biological activity. MIP-1β protein showed an increase of more than 300% in non-sevoflurane-LPS cells when compared with non-sevoflurane-PBS cells ($P < 0.005$). In the sevoflurane-LPS group, mRNA for MIP-1β was decreased by 24% at 5 h compared to the non-sevoflurane-LPS group ($P < 0.05$) (Fig. 2).

Upon LPS stimulation, MIP-2 mRNA increased rapidly in non-sevoflurane-exposed cells and reached a maximal expression after 1 h of LPS stimulation with an increase of 52% ($P < 0.005$). The addition of 1.1 vol % sevoflurane for 0.5 h was associated with a significant decrease of MIP-2 mRNA with 35% after 3 h, 49% after 5 h, and 131% after 7 h (all $P < 0.05$) (Fig. 3A).

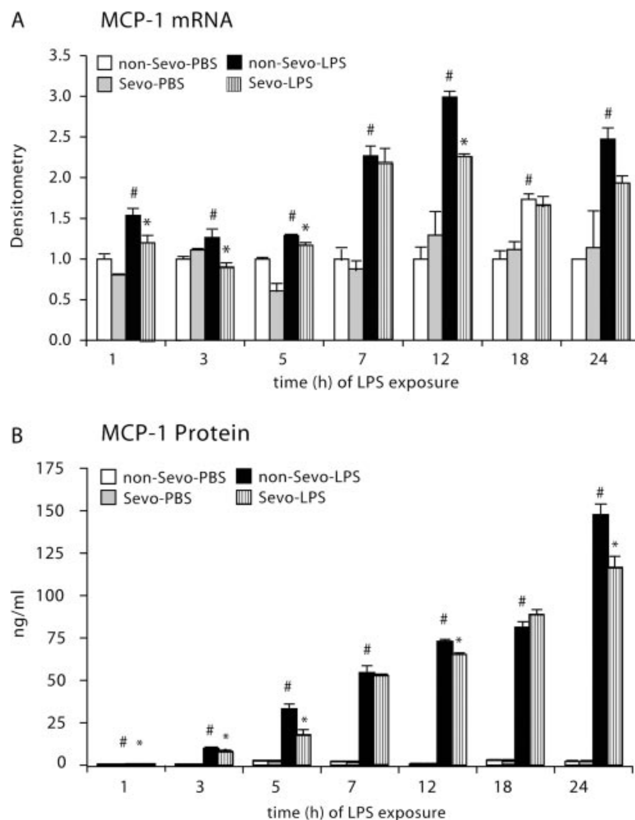

Figure 1. A: Evaluation of sevoflurane administration regarding mRNA expression for monocyte chemoattractant protein-1 (MCP-1). Confluent layers of alveolar epithelial cells (AEC) were pretreated with 1.1 vol % sevoflurane for 0.5 h (or with a control gas), followed by stimulation with lipopolysaccharide (LPS, 20 μg/mL) or phosphate-buffered saline (PBS) as a control for 1, 3, 5, 7, 12, 18, and 24 h. Total cellular mRNA was extracted and MCP-1 mRNA was determined by and reverse transcriptase-polymerase chain reaction (RT-PCR). Densitometry was performed and values adapted to 18S. One value for non-Sevo-PBS was defined as 1 and all other values were adapted. Values are mean ± SEM from five experiments. $^{\#}P < 0.05$ between non-Sevo-PBS and non-Sevo-LPS, $^{*}P < 0.05$ between non-Sevo-LPS and Sevo-LPS. B: Evaluation of sevoflurane administration regarding protein expression of monocyte chemoattractant protein-1 (MCP-1). Confluent layers of AEC were pretreated with 1.1 vol % sevoflurane for 0.5 h (or with a control gas), followed by stimulation with lipopolysaccharide (LPS, 20 μg/mL) or phosphate-buffered saline (PBS) as a control for 1, 3, 5, 7, 12, 18, and 24 h. MCP-1 ELISA was performed with supernatants. Values are mean ± SEM from five experiments. $^{\#}P < 0.05$ between non-Sevo-PBS and non-Sevo-LPS, $^{*}P < 0.05$ between non-Sevo-LPS and Sevo-LPS.

Only small amounts of MIP-2 protein were measured in the supernatant. After LPS stimulation, MIP-2 increased by 29% after 5 h ($P < 0.05$) and by 75% after 7 h ($P < 0.01$). MIP-2 was decreased by 22% after 7 h, exposing cells to 1.1 vol % sevoflurane before endotoxin stimulation ($P < 0.05$) (Fig. 3B).

CINC-1 mRNA was determined after 1, 3, 5, and 7 h of LPS stimulation time after a prior administration of 1.1 vol % sevoflurane or control gas for 0.5 h. LPS stimulation led to a maximum increase of CINC-1 mRNA of 400% after 5 h ($P < 0.001$). Treatment with

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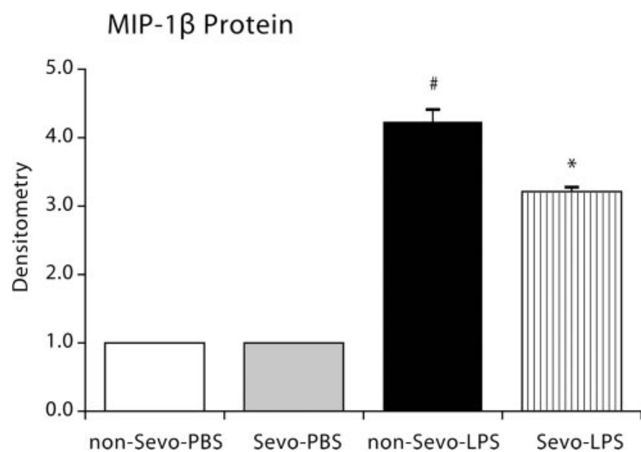

Figure 2. Evaluation of sevoflurane administration regarding protein expression of macrophage inflammatory protein-1β (MIP-1β). Confluent layers of alveolar epithelial cells (AEC) were pretreated with 1.1 vol % sevoflurane for 0.5 h (or with a control gas), followed by stimulation with lipopolysaccharide (LPS, 20 μg/mL) or phosphate-buffered saline (PBS) as a control for 5 h. MIP-1β was examined using Western blot analysis. Densitometry was performed. One value for non-Sevo-PBS was defined as 1 and all other values were adapted. Values are mean ± SEM from five different assays (no SEM for non-Sevo-PBS and sevo-PBS values). $^{\#}P < 0.005$ between non-Sevo-PBS and non-Sevo-LPS, $^{*}P < 0.05$ between non-Sevo-LPS and Sevo-LPS.

1.1 vol % sevoflurane was associated with a decrease of 46% at 1 h when compared with the non-sevoflurane-LPS group ($P < 0.005$) (Fig. 4A). After LPS stimulation, CINC-1 protein concentration increased from a baseline expression of 0.3 ± 0.0 ng/mL up to 2.9 ± 0.1 ng/mL ($P < 0.005$) at 3 h and 13.8 ± 0.4 ng/mL at 7 h ($P < 0.001$). Exposing AEC to sevoflurane before LPS stimulation decreased the CINC-1 concentration by 33% at 3 h, $P < 0.05$) and 20% at 5 h ($P < 0.05$) (Fig. 4B).

For ICAM-1 mRNA, a modest upregulation in non-sevoflurane-LPS stimulated cells was measured when compared with the non-sevoflurane-PBS group: 28% after 3 h, 50% at 5 h, and 32% at 7 h ($P < 0.05$). No statistically significant decrease of ICAM-1 mRNA was detected in the sevoflurane-LPS group (Appendix for Fig. 5 available at *www.anesthesia-analgesia.org*). This might have been due to a lack of sensitivity of the PCR method. A cell-based ELISA was performed to determine ICAM-1 protein on the surface of AEC, focusing on pretreatment with sevoflurane or control gas, followed by 5 h of LPS stimulation. There was a small but significant upregulation of ICAM-1 protein in non-sevoflurane LPS cells when compared with the non-sevoflurane PBS group (27% increase, $P < 0.001$). Thirty-six percent less ICAM-1 protein was found in the sevoflurane pretreated LPS group when compared with non-sevoflurane-LPS cells ($P < 0.05$) (Fig. 5).

Chemotaxis Assay

Chemotaxis assays were performed to assess the biological function of the released chemokines. The results showed a 68% ($P < 0.001$) increase of migrated PMN to the supernatant of LPS stimulated cells (5 h LPS stimulation) when compared with the supernatants of control cells. Chemotaxis of neutrophils to the supernatant of LPS-stimulated cells, previously exposed to 1.1 vol % sevoflurane for 0.5 h, decreased by 33% ($P < 0.05$) (Fig. 6A).

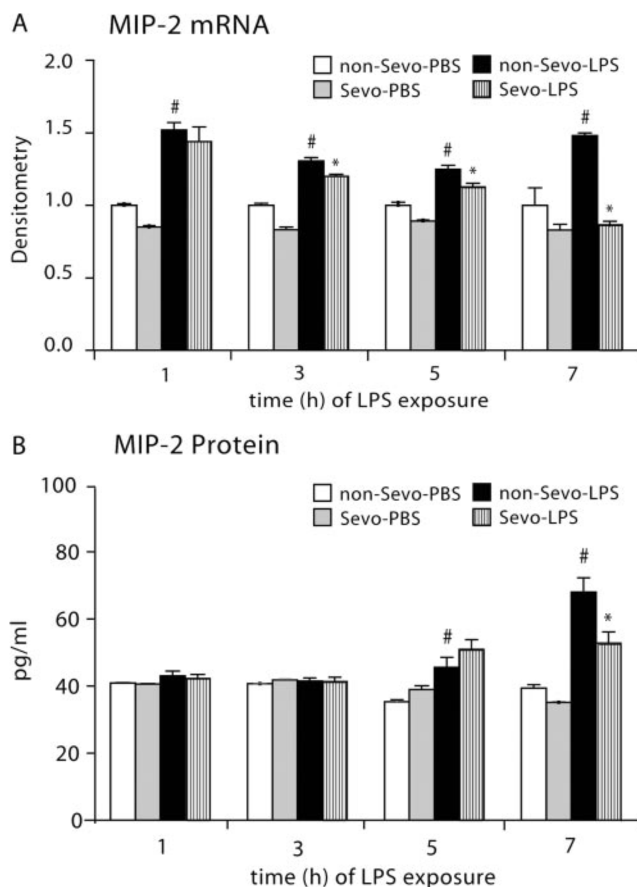

Figure 3. A: Evaluation of sevoflurane administration regarding mRNA expression for macrophage inflammatory protein-2 (MIP-2). Confluent layers of alveolar epithelial cells (AEC) were pretreated with 1.1 vol % sevoflurane for 0.5 h (or with a control gas), followed by stimulation with lipopolysaccharide (LPS, 20 μg/mL) or phosphate-buffered saline (PBS) as a control for 1, 3, 5, and 7 h. Total cellular mRNA was extracted and MIP-2 mRNA was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Densitometry was performed and values adapted to 18S. One value for non-Sevo-PBS was defined as 1 and all other values were adapted. Values are mean ± SEM from five experiments. $^{\#}P < 0.05$ between non-Sevo-PBS and non-Sevo-LPS, $^{*}P < 0.05$ between non-Sevo-LPS and Sevo-LPS. B: Evaluation of sevoflurane administration regarding protein expression of monocyte chemoattractant protein-1 (MIP-2). Confluent layers of AEC were pretreated with 1.1 vol % sevoflurane for 0.5 h (or with a control gas), followed by stimulation with lipopolysaccharide (LPS, 20 μg/mL) or PBS as a control for 1, 3, 5, and 7 h. MIP-2 ELISA was performed with supernatants. Values are mean ± SEM from five experiments. $^{\#}P < 0.05$ between non-Sevo-PBS and non-Sevo-LPS, $^{*}P < 0.05$ between non-Sevo-LPS and Sevo-LPS.

Adherence Assay

To evaluate the biological implication of ICAM-1 expression on AEC, adherence assays with neutrophils were performed. Compared to non-sevoflurane

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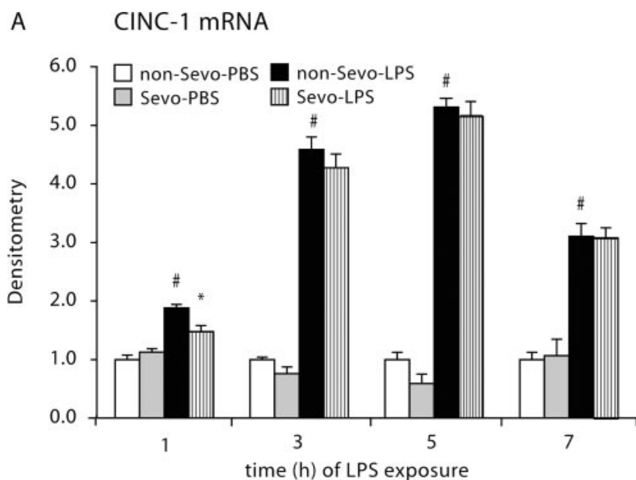

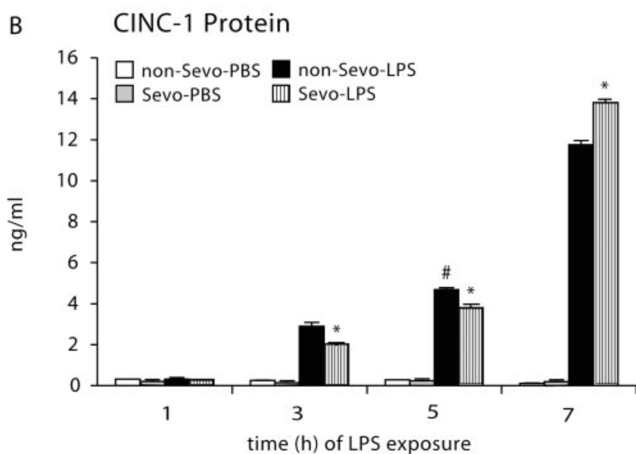

Figure 4. A: Evaluation of sevoflurane administration regarding mRNA expression for cytokine-induced neutrophil chemoattractant-1 (CINC-1). Confluent layers of alveolar epithelial cells (AEC) were pretreated with 1.1 vol % sevoflurane for 0.5 h (or with a control gas), followed by stimulation with lipopolysaccharide (LPS, 20 μg/mL) or phosphate-buffered saline (PBS) as a control for 1, 3, 5, and 7 h. Total cellular mRNA was extracted and CINC-1 mRNA was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Densitometry was performed and values adapted to 18S. One value for non-Sevo-PBS was defined as 1 and all other values were adapted. Values are mean ± SEM from five experiments. $^{\#}P < 0.05$ between non-Sevo-PBS and non-Sevo-LPS, $^{*}P < 0.005$ between non-Sevo-LPS and Sevo-LPS. B: Evaluation of sevoflurane administration regarding protein expression of cytokine-induced neutrophil chemoattractant-1 (CINC-1). Confluent layers of AEC were pretreated with 1.1 vol % sevoflurane for 0.5 h (or with a control gas), followed by stimulation with lipopolysaccharide (LPS, 20 μg/mL) or phosphate-buffered saline (PBS) as a control for 1, 3, 5, and 7 h. CINC-1 ELISA was performed with supernatants. Values are mean ± SEM from five experiments. $^{\#}P < 0.05$ between non-Sevo-PBS and non-Sevo-LPS, $^{*}P < 0.05$ between non-Sevo-LPS and Sevo-LPS.

PBS cells, adherence of neutrophils to non-sevoflurane LPS AEC increased by 78% ($P < 0.001$). Neutrophil adherence to sevoflurane-LPS cells decreased by 47% ($P < 0.001$). In the presence of blocking antibodies to ICAM-1, neutrophil adherence to non-sevoflurane-LPS cells was decreased by 38% ($P < 0.001$), and in sevoflurane-LPS cells by 14% ($P < 0.05$) (Fig. 6B).

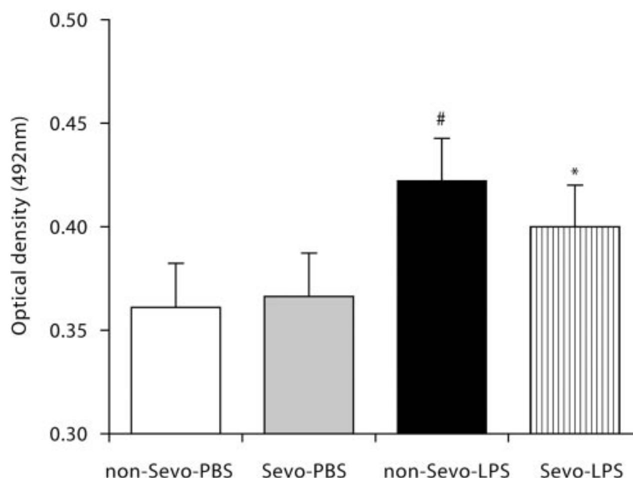

Figure 5. Evaluation of sevoflurane administration regarding protein expression of intercellular adhesion molecule-1 (ICAM-1). Confluent layers of alveolar epithelial cells (AEC) were pretreated with 1.1 vol % sevoflurane for 0.5 h (or with a control gas), followed by stimulation with lipopolysaccharide (LPS, 20 μg/mL) or phosphate-buffered saline (PBS) as a control for 5 h. Monolayers were fixed, and ICAM-1 was determined by direct cellular ELISA. Values are mean ± SEM from five experiments. $^{\#}P < 0.001$ between non-Sevo-PBS and non-Sevo-LPS, $^{*}P < 0.05$ between non-Sevo-LPS and Sevo-LPS.

DISCUSSION

LPS in the lung has been shown to trigger the release of a number of mediators, such as chemokines, cytokines, and cell adhesion molecules. These events combine to aid in the migration and activation of effector cells, such as alveolar macrophages and PMN cells (15). AEC are a potent source of inflammatory mediators within the respiratory compartment of the lung upon endotoxin stimulation (16). Sevoflurane, a newer volatile anesthetic, has antiinflammatory properties in the heart, as previously demonstrated (13). Because AEC are directly exposed to volatile anesthetics, this study investigated potential inflammation-modifying effects of sevoflurane in a model of ALI. The main results of our study showed downregulation of chemokines and adhesion molecules, released by AEC upon LPS stimulation after pretreatment with sevoflurane, with the concomitant biological consequences.

Pharmacological preconditioning is a powerful cell-protective mechanism conferring relative resistance against myocellular death resulting from ischemia-reperfusion injury (13). Some volatile anesthetics also protect against ischemia-reperfusion injury in kidneys by attenuating inflammation (17). A study in rat alveolar Type II cells showed that exposure to volatile anesthetics altered secretion of inflammatory mediators upon interleukin-1β stimulation (18). Halothane, isoflurane, and enflurane decreased production of interleukin-6, MIP-2, and MCP-1 protein concentrations in a dose- and time-dependent manner. Other *in*

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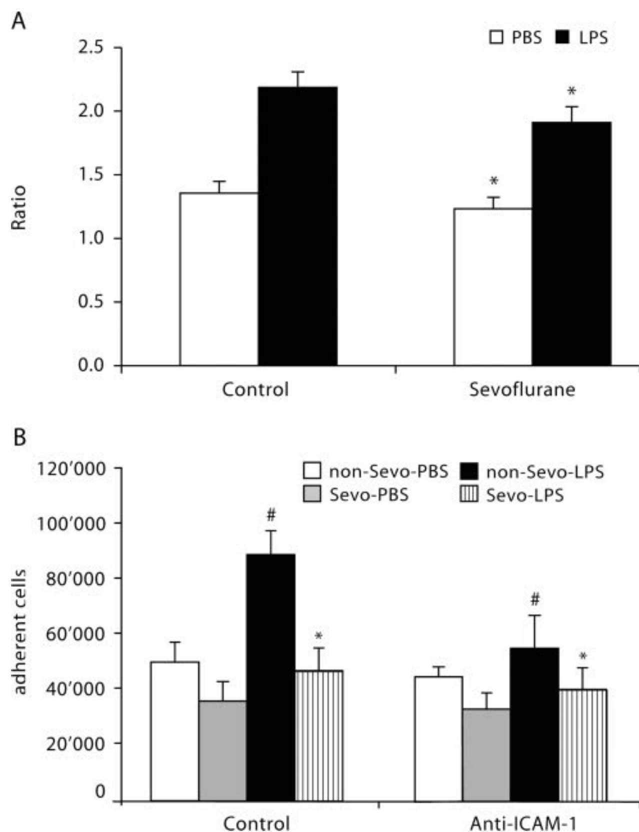

Figure 6. A: Determination of chemotactic activity in supernatants of alveolar epithelial cells (AEC). Calcein-AM-labeled neutrophils were given into MultiScreen-MIC filter plates, while receiver plates were loaded with 150 μL of supernatant of previously sevoflurane/non-sevoflurane pretreated phosphate-buffered saline (PBS)- or lipopolysaccaride (LPS)-exposed cells. Dulbecco's modified Eagle's medium (DMEM) 1% fetal bovine serum (FBS) was taken to determine basal migration. After 2 h of incubation, fluorescence was measured by using an excitation filter at 485 nm and an emission filter at 535 nm. Ratio between migration to supernatant of stimulated AEC and basal migration was used as indication of PMN chemotaxis. Values are mean ± SEM from five experiments. $^{*}P < 0.05$ between non-Sevo-PBS and Sevo-PBS as well as between non-Sevo-LPS and Sevo-LPS. B: Neutrophil adhesion to AEC cell monolayers. Cells were exposed to sevoflurane or control gas and stimulated with LPS for 5 h. Cells were preincubated with anti-ICAM-1 (1A29) or control antibody (MOPC-21). At the same time, neutrophils were preincubated with anti-FcγRIII (CD16) and anti-FcγRII (CD32) antibodies × calcein-AM-labeled PMN were added to each well for 30 min. Nonadherent PMN were removed by carefully washing the cells twice with DMEM. Fluorescence was measured by using an excitation filter at 485nm and an emission filter at 535nm. The amount of adherent PMN was calculated by using a standard row. Values are mean ± SEM from five experiments. $^{\#}P < 0.05$ between non-Sevo-PBS and non-Sevo-LPS, $^{*}P < 0.05$ between non-Sevo-LPS and Sevo-LPS.

vitro studies demonstrated that halothane altered surfactant phospholipids and apoprotein biosynthesis by rat Type II AEC and decreased Na, K-ATPase-, and sodium channel activities (19,20). Our results with a sevoflurane-induced downregulation of inflammatory mediators are in accordance with these previous findings, although the experimental setup was different. They suggest that this sevoflurane-induced inhibiting effect in LPS-induced lung injury may be dose-dependent within a range of 1.1–2.2 vol% sevoflurane, a concentration which is also clinically applicable. Comparing our findings with results from Giraud et al. (18), testing halothane, or with Kehl et al. (21), working with isoflurane, it seems that the threshold concentration required for a certain effect may be specific for a given volatile anesthetic.

In contrast to these findings, several studies have revealed that the use of volatile anesthetics modulate expression of inflammatory mediators into the opposite direction. Kotani et al. (22) found that the exposure of rat alveolar macrophages to volatile anesthetics augmented mRNA expression of proinflammatory cytokines. Sevoflurane also increased pulmonary NO_3 and NO_2 production in anesthetized pigs (23). A possible explanation of these contradictory results might be the differences in experimental setups, the use of several volatile anesthetics, the concentration of the volatile anesthetic, and the time of exposure.

In our model, important PMN chemoattractants, such as MIP-2 and CINC-1, were determined. MCP-1 was chosen because it is not only as a monocyte but also as a neutrophil chemoattractant. MCP-1 has been shown to play a pivotal role in PMN recruitment in various experimental systems: MCP-1 was demonstrated to be important for neutrophil recruitment in hyperoxia-exposed rat (24). Also, studies in a model of LPS-induced lung injury showed that MCP-1 is involved in accumulation of neutrophils (25).

The biological aspect of this study is noteworthy: we could show that sevoflurane suppresses the expression of inflammatory mediators with subsequent important biological relevance. Because chemokines are implicated in effector cell recruitment toward target tissues, the effect of sevoflurane on LPS-stimulated AEC was investigated performing chemotaxis assays. LPS stimulation of AEC induced enhanced chemotaxis. Notably, with sevoflurane pretreatment, chemotactic potency was decreased. These results suggest that sevoflurane reduces AEC-induced accumulation of neutrophils in LPS injury.

Increased adhesiveness between pneumocytes and neutrophils in the setting of an inflammatory response would be expected to lead to intensified injury of the alveolar cell-lining barrier (11,26). Results of the adherence assays demonstrated a functional role for ICAM-1 in the adhesion of neutrophils to LPS-stimulated AEC. With sevoflurane, decreased adhesion of these effector cells to AEC could be shown, assuming that epithelial cell killing might also be impaired. This would imply a diminished grade of injury.

In summary, we demonstrated that sevoflurane pretreatment decreased the production of inflammatory mediators in AEC in LPS-induced injury. The advantage of the respiratory compartment is the abundance of available tissue for analysis of inflammatory reactions at the interface of the external and internal

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environments. The lung offers easy accessibility for therapeutic interventions. Attenuation of injury by application of sevoflurane in the ALI could be a promising option for clinical application. However, exact information about cellular signaling upon interaction of sevoflurane with the target cell has to be evaluated.

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