Anesthesiology 2006; 104:961–9

Ropivacaine Decreases Inflammation in Experimental Endotoxin-induced Lung Injury

Stephan Blumenthal, M.D., Alain Borgeat, M.D.,† Thomas Pasch, M.D.,‡ Livia Reyes,§ Christa Booy,§ Maud Lambert, M.D.,‖ Ralph C. Schimmer, M.D., M.B.A.,# Beatrice Beck-Schimmer, M.D.***

Background: **Endotoxin causes acute lung injury, which can lead to acute respiratory distress syndrome. Because local anesthetics are known to attenuate inflammatory reactions, ropivacaine was tested for its possible antiinflammatory effect in lipopolysaccharide-induced lung injury in rat alveolar epithelial cells (AECs) and rat pulmonary artery endothelial cells (RPAECs) *in vitro* and *in vivo*.**

Methods: **AECs and RPAECs were stimulated for 4 h with lipopolysaccharide or lipopolysaccharide and 1 μM ropivacaine. Messenger RNA (mRNA) for intercellular adhesion molecule 1 was assessed. Isolated neutrophils were incubated with stimulated target cells to quantify adhesion and neutrophil-induced cytotoxicity in AECs and RPAECs. *In vivo*, lipopolysaccharide was instilled intratracheally with or without 1 mM intratracheally or intravenously administered ropivacaine. Bronchoalveolar lavage was performed 5 h later to determine neutrophil and albumin content, as well as concentrations of inflammatory mediators.**

Results: **In AECs and RPAECs, ropivacaine attenuated lipopolysaccharide-induced up-regulation of mRNA for intercellular adhesion molecule 1 by 41% and 24%, respectively ($P < 0.05$). In the presence of ropivacaine, increased neutrophil adhesion was down-regulated by 58% and 44% ($P < 0.005$), whereas cytotoxicity in AECs and RPAECs was diminished by 28% and 33%, respectively ($P < 0.05$). Enhanced neutrophil count in lipopolysaccharide lungs was reduced by 56% in the presence of intratracheally instilled ropivacaine (81% with intravenous ropivacaine; $P < 0.005$). Albumin was decreased by 46% with intratracheal ropivacaine (38% with intravenous ropivacaine; $P < 0.05$), and inflammatory mediators were decreased by 48–59% (69–81% with intravenous ropivacaine; $P < 0.01$).**

Conclusions: **Ropivacaine intervention substantially attenuated the inflammatory response in acute lung injury and thus may carry an interesting potential for antiinflammatory treatment.**

INTRATRACHEAL application of bacterial endotoxin leads to an inflammatory reaction with pathologic characteristics, resembling an acute respiratory distress syndrome. This acute lung injury (ALI) represents a suitable experimental system to investigate the immunopathologic mechanisms of acute respiratory distress syndrome. Studies using lipopolysaccharide to induce lung injury have mainly focused on interactions involving the vascular compartment; however, interest in the epithelial compartment has increased since its contribution to the inflammatory reaction by interaction of effector cells (macrophages, neutrophils) with target cells (airway epithelial cells) has been shown.[1–3] A local intervention through the airways is a possible diagnostic and therapeutic option.

Both rat pulmonary artery endothelial cells (RPAECs) and alveolar epithelial cells (AECs) express and secrete various inflammatory mediators, such as adhesion molecules (intercellular adhesion molecule 1 [ICAM-1]), cytokines (tumor necrosis factor α [TNF-α]) and chemokines (cytokine-induced neutrophil chemoattractant 1 [CINC-1], monocyte chemoattractant protein 1 [MCP-1]).[3–7] The airway epithelial is thought to play an important role in the initiation and exacerbation of inflammatory response within the airways, recruiting effector cells through the expression and production of these inflammatory mediators.

Local anesthetics have been shown to have antiinflammatory properties, influencing a broad spectrum of effector cell functions such as phagocytosis and superoxide production, which are relevant to inflammatory diseases.[8,9] Ropivacaine, a newer local anesthetic, shows many of the properties of other highly liposoluble local anesthetics but benefits from a larger safety margin.[10–12] Few studies exist regarding the possible protective effects of this local anesthetic in inflammatory situations.[13]

We hypothesized decreased inflammatory reactions and attenuation of ALI in the presence of ropivacaine. To explore this research questions, the immune modulating effect of ropivacaine was evaluated in a model of lipopolysaccharide-induced lung injury in rat AECs and RPAECs (*in vitro*) and in rat lung (*in vivo*).

Materials and Methods

Animals

All animal experiments and animal care were approved by the Swiss Veterinary Health Authorities, Zurich, Switzerland.

* Assistant Professor, Department of Anesthesiology, Orthopedic University Clinic Zurich Balgrist. † Professor, Department of Anesthesiology, Orthopedic University Clinic Zurich Balgrist. ‖ Resident, Department of Anesthesiology, Orthopedic University Clinic Zurich Balgrist. ‡ Professor and Chairman, Institute of Anesthesiology, § Technical Assistant, Institute of Physiology, # Associate Professor, Department of Surgery, ** Associate Professor, Institutes of Anesthesiology and Physiology, University of Zurich.

Received from the Institutes of Anesthesiology and Physiology, University of Zurich, Zurich, Switzerland, and the Department of Anesthesiology, Orthopedic University Clinic Zurich, Balgrist, Switzerland. Submitted for publication August 18, 2005. Accepted for publication November 30, 2005. Supported by the Research Foundation of the Swiss Society of Anesthesiology and Resuscitation, Berne, Switzerland. Presented at the Annual Meeting of Euroanaesthesia, Glasgow, Scotland, June 2, 2003, and at the Annual Meeting of the Swiss Society of Anesthesiology and Resuscitation 2003, Interlaken, Switzerland, November 7, 2003.

Address correspondence to Dr. Beck-Schimmer: Institute of Anesthesiology, Institute of Physiology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland. beatrice_beck.schimmer@access.unizh.ch.

Table 1. Optimized Conditions for Reverse-transcription Polymerase Chain Reaction for Messenger RNA

Gene	Primers	Thermocycle Condition	Temperature, °C
ICAM-1			
Sense	5′-AGG TAT CCA TCC ATC CCA CA-3′	AECs, 27 cycles	58
Antisense	5′-CTT CAG AGG CAG GAA ACA GG-3′	RPAECs, 24 cycles	58
18S			
Sense	5′-TGA GGC CAT GAT TAA GAG GG-3′	AECs, 24 cycles	57
Antisense	5′-AGT CGG CAT CGT TTA TGG TC-3′	RPAECs, 22 cycles	57

AEC = alveolar epithelial cell; ICAM-1: intercellular adhesion molecule 1; RPAEC = rat pulmonary artery endothelial cell.

Alveolar Epithelial Cells

L2 cells (CCL 149; ATCC, Rockville, MD), a clonally isolated cell line from the adult rat lung of alveolar epithelial cell type II origin, were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% HEPES buffer.[14] Experiments were performed with confluent cells from passages 10–20. Before cell stimulation, the medium was changed to DMEM–1% FBS.

Rat Pulmonary Artery Endothelial Cells

Rat pulmonary artery endothelial cells, kindly provided by Roscoe Warner, Ph.D. (Research Assistant Professor, Department of Pathology, University of Michigan, Ann Arbor, Michigan), were cultured in DMEM, supplemented with 10% FBS, 1% penicillin, and 1% HEPES. RPAECs from passages 25–35 were grown until confluence.

Stimulation with Lipopolysaccharide

Confluent monolayers of AECs and RPAECs were exposed to *Escherichia coli* serotype 055:B5 lipopolysaccharide (20 μg/ml; Sigma, Buchs, Switzerland) for 4 h, diluted in DMEM–1% FBS, or to phosphate-buffered saline (PBS) in DMEM-1% FBS as a control.

In a first experimental setup, different blocking concentrations (100, 10, and 1 μM) of ropivacaine (AstraZeneca, Zug, Switzerland) were tested for the effect of ICAM-1 expression in AECs and RPAECs. Lipopolysaccharide and ropivacaine were added at the same time to the cells. One micromolar proved to be the most effective concentration. For the final experiments, four different groups were designed: PBS–PBS, PBS–1 μM ropivacaine, lipopolysaccharide–PBS, and lipopolysaccharide–1 μM ropivacaine. Cells were collected after 4 h of stimulation. Ropivacaine-induced cytotoxicity was excluded performing lactate dehydrogenase assays (Promega, Madison, WI).

RNA Extraction and Reverse-transcription Polymerase Chain Reaction

Total RNA from AECs and RPAECs was extracted using TRIZOL® (Invitrogen, Life Technologies, Carlsbad, CA) as described previously, and messenger RNA (mRNA) for ICAM-1 was determined.[15] For analysis, reverse-transcription polymerase chain reaction was performed using a polymerase chain reaction core kit (Applied Biosystems, Rotkreuz, Switzerland). Random hexanucleotide primers and murine leukemia virus reverse transcriptase were used for complementary DNA synthesis. Reverse transcription was performed with 0.8 μg RNA at 20°C for 5 min, at 42°C for 30 min, and at 99°C for 5 min. Primers located on separate exons were designed to assess expression ICAM-1 (table 1). For the primer pair, optimal cycling conditions were tested to detect target genes within the logarithmic amplification phase. Reverse-transcription polymerase chain reaction products were resolved on 1.5% agarose gels (Ultra Pure; Invitrogen, Basel, Switzerland) and stained with ethidium bromide.

Isolation of Neutrophils

Venous blood was obtained from healthy adult volunteers and anticoagulated with citrate–dextrose solution (Sigma, Buchs, Switzerland) as described previously.[16] Neutrophils were isolated by gradient centrifugation over Ficoll-Paque (Amersham Pharmacia, Biotech, Dubendorf, Switzerland) followed by 1% dextran (Amersham Pharmacia, Biotech, Dubendorf, Switzerland) sedimentation for 1 h to separate neutrophils from erythrocytes. After centrifugation of the supernatant, the contaminating erythrocytes were lysed. The remaining neutrophils were resuspended in Hank's balanced salt solution without calcium and magnesium (Life Technologies, Basel, Switzerland). The entire procedure was performed at 15°C.

Assay of Neutrophil Adherence

Alveolar epithelial cells or RPAECs were plated in DMEM–10% FBS on tissue culture–treated 96-well microtiter plates (NUNC Products, Life Technologies, Basel, Switzerland) and grown to confluence at 37°C with 5% CO_2. After stimulation with *E. coli* serotype 0.55:B5 lipopolysaccharide (20 μg/ml) in DMEM–1% FBS or a mixture of lipopolysaccharide and 1 μM ropivacaine for 4 h, 1×10^6 neutrophils were added to each well, and the microtiter plates were incubated for 15 min at 37°C with 5% CO_2. This incubation step allowed neutrophils to settle onto the epithelial cell monolayer before being stimulated with phorbol 12-myristate 13-acetate (final concentration of 100 ng/well). Thirty minutes later, non-

adherent cells were removed by washing twice with DMEM. Finally, the number of adherent neutrophils was determined by counting them in a defined area using a Neubauer chamber.

Cytotoxicity Assay

Cytotoxicity was measured using a nonradioactive standard lactate dehydrogenase (LDH) assay (Promega). AECs or RPAECs were seeded into the wells of a 96-well culture dish. Cells were stimulated with lipopolysaccharide (20 μg/ml) for 4 h. Neutrophils (1×10^6) in PBS–0.02% bovine serum albumin were added to each well, followed by a stimulation with phorbol 12-myristate 13-acetate 15 min later as described. After incubation for 5 h, the supernatant was removed from each well and centrifuged at 700*g* for 5 min. Some target cells were incubated with lysing solution for 45 min (target cell maximum LDH release). Supernatant was also collected from wells of AECs or RPAECs, which had not been incubated with neutrophils (spontaneous LDH release). Supernatant from wells, incubated with neutrophils, were defined as experimental release. LDH content was determined by measuring red formazan resulting from the conversion of tetrazolium salt into this substrate by LDH. Cytotoxicity was calculated according to the following formula:

$$\text{Cytotoxicity } \% = \frac{\text{experimental release} - \text{spontaneous release}}{\text{target cell maximum release} - \text{spontaneous release}} \times 100.$$

Animal Model of Acute Lung Injury

Pathogen-free male Wistar rats (250–300 g) were housed in individual isolator cages within the Animal Care Facilities at the University of Zurich. Rats were anesthetized with subcutaneously administered Hypnorm® (fentanyl–fluanisone; Janssen, Beerse, Belgium), 0.25 ml/kg body weight; Domitor® (medetomidine; Pfizer, Inc., Westchester, PA), 0.25 ml/kg body weight; and 0.1% atropine, 0.05 ml/kg body weight.

The lowest concentration of ropivacaine with best effect was determined in pilot experiments (data not shown). In a first series of experiments, lung injury was induced by intratracheal instillation of 150 μg lipopolysaccharide in 300 μl PBS either with or without 1 mM ropivacaine. For intravenous blocking experiments, which were performed in a second series, 300 μl of 1 mM ropivacaine or 300 μl PBS for control animals were applied intravenously immediately after intratracheal lipopolysaccharide instillation. In both series, four groups were tested (PBS–PBS, PBS–ropivacaine, lipopolysaccharide–PBS, and lipopolysaccharide–ropivacaine), and every group consisted of five animals. Five hours after onset of lung injury, animals were killed, and bronchoalveolar lavage was performed, followed by excision of the lung.

Bronchoalveolar Lavage

For bronchoalveolar lavage, 10 ml PBS was gently instilled into the lungs, withdrawn, and reinstilled three times and collected. Bronchoalveolar lavage fluid (BALF) was centrifuged at 700*g*. Supernatant was aliquoted and frozen at −20°C. Cell pellets from centrifuged BALF were assessed for differential cell counts using cytospins and Diff-Quick (Dade Behring, Duedingen, Switzerland).

Determination of Inflammatory Mediators in BALF

To study inflammatory changes in the respiratory compartment, the following mediators were determined: TNF-α, CINC-1, and MCP-1. Enzyme-linked immunosorbent assays (ELISAs) were performed according to the manufacturer's recommendations, using OptEIA rat TNF-α and MCP-1 ELISA (Pharmingen, San Diego, CA) and rat CINC-1 ELISA (R&D Systems, Abingdon, United Kingdom), respectively. Optical density was determined at 492 nm.

Albumin Extravasation

Extravasation of albumin was assessed by a direct ELISA according to a previous protocol.[17] Briefly, coating carbonate buffer (0.1 M carbonate, pH 9.5) was used to dilute samples (BALF from injured lungs, 1:1,000), and a standard curve was created with recombinant rat albumin (RDI, Flanders, NJ). Ninety-six–well plates were coated with samples and recombinant albumin and incubated overnight at 4°C. A first polyclonal rabbit anti-rat albumin antibody (RDI; 10 μg/ml) was incubated for 1 h at 4°C (rabbit immunoglobulin G used as a control), followed by a 1-h incubation at 4°C with a secondary horseradish peroxidase–labeled goat anti-rabbit antibody (Sigma, Buchs, Switzerland). Color reaction was developed with *o*-phenylenediamine dihydrochloride (Sigma), the reaction was stopped with 3 M H_2SO_4, and optical density was determined at 492 nm (ELISA reader; Bioconcept, Allschwil, Switzerland).

Statistical Analysis

Results are expressed as mean ± SEM. Increases were expressed in percentage or absolute values. Ropivacaine-induced reduction in the presence of lipopolysaccharide was calculated as the relative change compared with lipopolysaccharide without ropivacaine results (= 100%). All experiments were conducted at least five times. Analysis of variance with post–analysis of variance comparison was performed to assess the statistical significance of differences at a 5% level. Densitometry data are expressed as percentage values of controls (control = 100%).

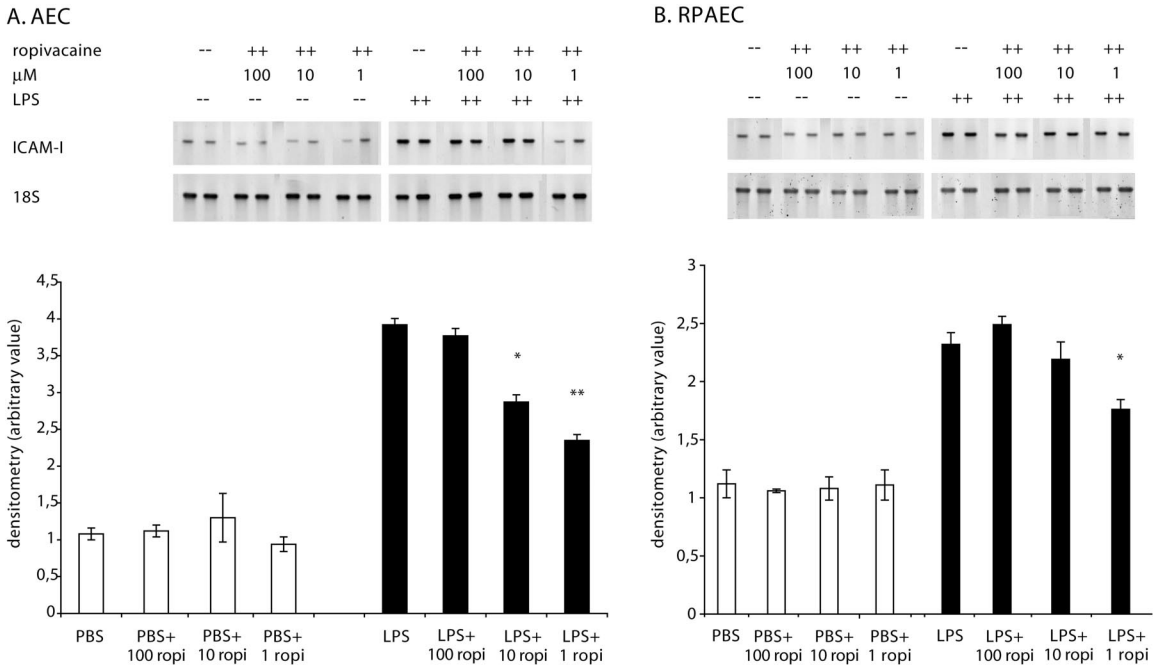

Fig. 1. (*A*) Determination of messenger RNA (mRNA) of intercellular adhesion molecule 1 (ICAM-1) in alveolar epithelial cells (AECs). Monolayers of cells were exposed to lipopolysaccharide (LPS) (or phosphate-buffered saline [PBS] as a control) for 4 h. The effect of different concentrations of ropivacaine (ropi; 100, 10, and 1 μM) were evaluated. *White bars* represent values from control cells; *black bars* represent values from after lipopolysaccharide stimulation. Results in densitometry are expressed as percentage of values obtained from control cells (= 1.0). Values are mean ± SEM from five different assays. The blot represents one of five experiments. * $P < 0.05$, ** $P < 0.01$. (*B*) Determination of mRNA of ICAM-1 in rat pulmonary artery endothelial cells (RPAECs). Monolayers of cells were exposed to lipopolysaccharide (or PBS as a control) for 4 h. The effect of different concentrations of ropivacaine (100, 10, and 1 μM) were evaluated. Results in densitometry are expressed as percentage of values obtained from control cells (= 1.0). Values are mean ± SEM from five different assays. The blot represents one of five experiments. * $P < 0.05$.

Results

Ropivacaine-induced Change of ICAM-1 mRNA Expression in AECs and RPAECs

Alveolar epithelial cells were stimulated with lipopolysaccharide for 4 h with or without the presence of ropivacaine. Control cells were exposed to PBS. Several concentrations of this local anesthetic were tested in a first experimental setup to assess the blocking effect of ropivacaine: 100, 10, and 1 μM final concentration of ropivacaine. RNA was extracted, and reverse-transcription polymerase chain reaction was performed. Upon lipopolysaccharide stimulation, an up-regulation of mRNA for ICAM-1 of 263% could be demonstrated compared with control cells ($P < 0.005$; fig. 1A). Coincubation of the cells with the local anesthetic attenuated increased ICAM-1 expression according to the used ropivacaine concentration. Whereas 100 μM ropivacaine did not show any blocking effect, 10 μM ropivacaine down-regulated ICAM-1 expression by 27% ($P < 0.05$). One micromolar ropivacaine as the most effective concentration had an inhibitory effect showing a decrease of ICAM-1 expression by 41% ($P < 0.01$).

Changes in mRNA for ICAM-1 showed a similar pattern in RPAECs as demonstrated in AECs (fig. 1B). Lipopolysaccharide enhanced ICAM-1 expression with an increase of 107% ($P < 0.05$). Ropivacaine at 100 and 10 μM did not significantly alter mRNA for ICAM-1, but 1 μM ropivacaine decreased up-regulated ICAM-1 expression in endothelial cells by 24% ($P < 0.05$).

Adherence of Neutrophils to Alveolar Epithelial and Pulmonary Endothelial Cells after Lipopolysaccharide Exposure: Role of Ropivacaine

Adherence assays were performed using neutrophils to define the biologic role of ropivacaine in regard to up-regulated ICAM-1 (fig. 2A). AECs were exposed to lipopolysaccharide for 4 h, followed by an incubation with phorbol 12-myristate 13-acetate–stimulated neutrophils. Adherence of these effector cells to endotoxin-exposed cells increased substantially by 1,115% compared with control cells ($P < 0.00001$). Ropivacaine administration blocked this enhanced adherence by 58% ($P < 0.0001$).

In the case of RPAECs, increased adhesion of neutrophils (692% increase, $P < 0.0001$) was attenuated by 44% with ropivacaine ($P < 0.005$; fig. 2B).

Damage to Alveolar Epithelial and Pulmonary Endothelial Cells Caused by Neutrophils

We also studied the ability of ropivacaine to influence neutrophil-induced damage to AECs and RPAECs. Phorbol 12-myristate 13-acetate–stimulated neutrophils were

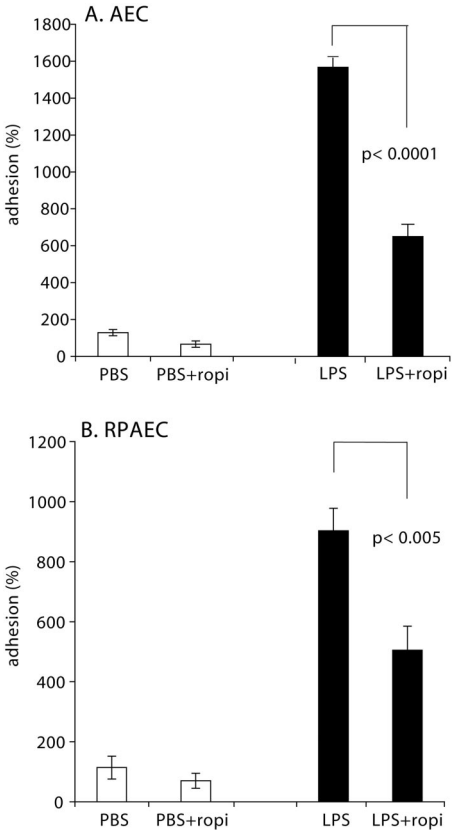

Fig. 2. (*A*) Neutrophil adhesion to alveolar epithelial cell (AEC) monolayers. AECs were exposed to lipopolysaccharide (LPS) or phosphate-buffered saline (PBS) with or without 1 μM ropivacaine (ropi) for 4 h. Neutrophils, 1 × 10^6, were added to each well for 15 min, followed by a stimulation with phorbol 12-myristate 13-acetate. After 30 min of incubation, nonadherent neutrophils were removed by carefully washing the cells twice with Dulbecco's modified Eagle's medium. The number of adherent cells was counted. Values are mean ± SEM from five experiments. (*B*) Neutrophil adhesion to rat pulmonary artery endothelial cell (RPAEC) monolayers. RPAECs were exposed to lipopolysaccharide or PBS with or without 1 μM ropivacaine for 4 h. Neutrophils, 1 × 10^6, were added to each well for 15 min, followed by a stimulation with phorbol 12-myristate 13-acetate. After 30 min of incubation, nonadherent neutrophils were removed by carefully washing the cells twice with Dulbecco's modified Eagle's medium. The number of adherent cells was counted. Values are mean ± SEM from five experiments.

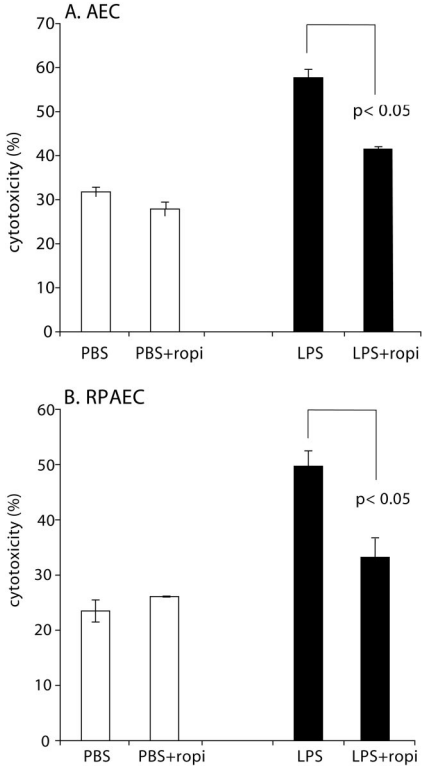

Fig. 3. (*A*) Determination of neutrophil-induced alveolar epithelial cell (AEC) killing. Lactate dehydrogenase release was measured as a measure for degree of cytotoxicity. AECs were stimulated with phosphate-buffered saline (PBS) or lipopolysaccharide (LPS) for 4 h with or without 1 μM ropivacaine (ropi), followed by an incubation with phorbol 12-myristate 13-acetate-stimulated neutrophils for 5 h. Cytotoxicity was calculated as described. Values are shown as mean ± SEM. The experiment was performed three times with similar results. (*B*) Determination of neutrophil-induced rat pulmonary artery endothelial cell (RPAEC) killing. Lactate dehydrogenase release was measured as a measure for degree of cytotoxicity. RPAECs were stimulated with PBS or lipopolysaccharide for 4 h with or without 1 μM ropivacaine, followed by an incubation with phorbol 12-myristate 13-acetate-stimulated neutrophils for 5 h. Cytotoxicity was calculated as described. Values are shown as mean ± SEM. The experiment was performed three times with similar results.

incubated with lipopolysaccharide-stimulated AECs or with control cells, and cytotoxicity was determined. Figure 3 shows LDH release as an indirect measurement of cell death. Neutrophils as a source of possible LDH release could be excluded by trypan blue staining. Control AECs exposed to stimulated neutrophils showed a cytotoxicity of 32%, whereas cytotoxicity of lipopolysaccharide-stimulated AECs increased to 58% ($P < 0.01$; fig. 3A). With ropivacaine administration, cytotoxicity was observed to decrease by 28% ($P < 0.05$).

Similar data were obtained with RPAECs (fig. 3B). Lipopolysaccharide induced an increase of cytotoxicity by 111% ($P < 0.05$). This increase was attenuated in the presence of ropivacaine by 33% ($P < 0.05$).

Effect of Ropivacaine on Neutrophil Accumulation in Lipopolysaccharide-injured Lungs

To evaluate cellular elements quantitatively and qualitatively in the respiratory compartment, BALF cells were analyzed, with focus on neutrophils. As seen in figure 4A, neutrophil content increased 5 h after lipopolysaccharide stimulation from 0.2 × 10^6 to 43 × 10^6 cells/ml ($P < 0.00001$). Intratracheal application of ropivacaine in lipopolysaccharide-injured lungs attenuated neutrophil accumulation by 56% ($P < 0.005$).

Intervention with intravenously applied ropivacaine reduced neutrophil recruitment by 81% (fig. 4B) compared with lipopolysaccharide–PBS animals ($P < 0.0001$).

Ropivacaine and Inflammatory Mediators in the Respiratory Epithelial Compartment

To determine possible neutrophil chemoattractants, responsible for the ropivacaine-induced attenuation of

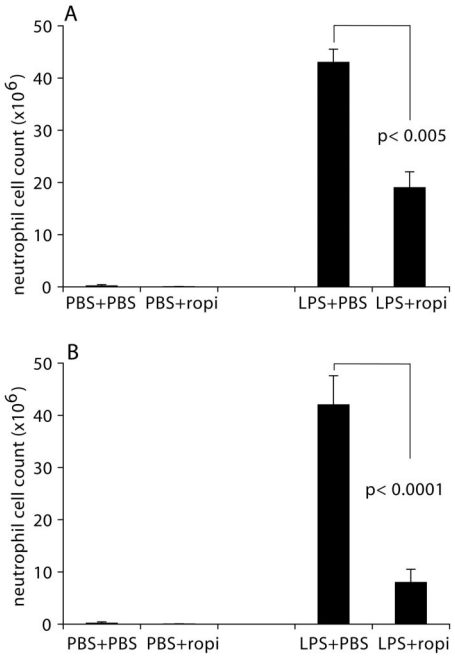

Fig. 4. (*A*) Total neutrophil cell count in bronchoalveolar lavage fluid after intratracheal application of ropivacaine (ropi). Lipopolysaccharide (LPS), 150 μg, in 300 μl phosphate-buffered saline (PBS) was intratracheally instilled (or PBS alone for control animals) in the presence or absence of 1 mM ropivacaine. Lungs were evaluated 5 h later. Cells were analyzed using cytospin and Diff-Quick staining. Values are mean ± SEM from five animals per group. (*B*) Total neutrophil cell count in bronchoalveolar lavage fluid after intravenous application of ropivacaine. Lipopolysaccharide, 150 μg, in 300 μl PBS was intratracheally instilled (or PBS alone for control animals). At the same time, 300 μl PBS with 1 mM ropivacaine (control animals with PBS only) was applied intravenously. Lungs were evaluated 5 h later. Cells were analyzed using cytospin and Diff-Quick staining. Values are mean ± SEM from five animals per group.

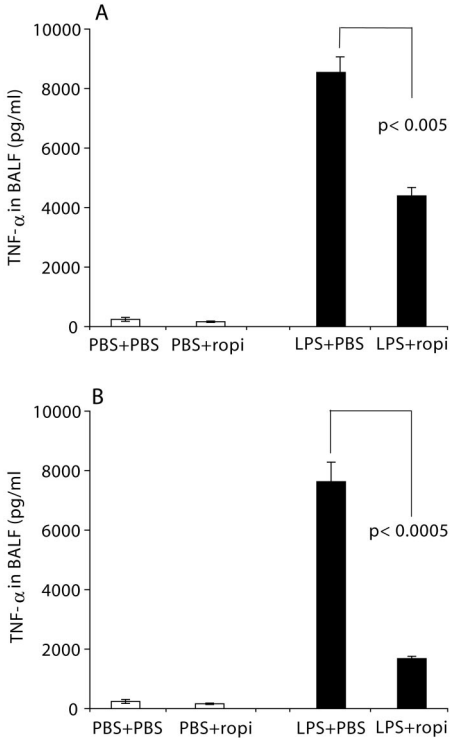

Fig. 5. (*A*) Tumor necrosis factor α (TNF-α) protein determination in bronchoalveolar lavage fluid (BALF) after intratracheal application of ropivacaine (ropi). Lipopolysaccharide (LPS), 150 μg, in 300 μl phosphate-buffered saline (PBS) was intratracheally instilled (or PBS alone for control animals) in the presence or absence of 1 mM ropivacaine. Bronchoalveolar lavage was performed 5 h later. TNF-α was examined using a standard enzyme-linked immunosorbent assay. Values are mean ± SEM from five animals. (*B*) TNF-α protein determination in BALF after intravenous application of ropivacaine. Lipopolysaccharide, 150 μg, in 300 μl PBS was intratracheally instilled (or PBS alone for control animals). At the same time, 300 μl of 1 mM ropivacaine in PBS was intravenously applied (control animals with PBS only). Bronchoalveolar lavage was performed 5 h later. TNF-α was examined using a standard enzyme-linked immunosorbent assay. Values are mean ± SEM from five animals.

neutrophil recruitment, protein expression of the important inflammatory mediators TNF-α, CINC-1, and MCP-1 in BALF was assessed.

Tumor necrosis factor α concentration increased from 0.24 ng/ml in PBS animals to 8.54 ng/ml in endotoxin-injured animals ($P < 0.00001$; fig. 5A). In the presence of intratracheally applied ropivacaine, TNF-α expression was attenuated by 48% ($P < 0.005$). With intravenous ropivacaine, TNF-α concentration was decreased by 78% ($P < 0.0005$; fig. 5B).

In PBS animals, a baseline CINC-1 concentration of 0.15 ng/ml was measured in the respiratory compartment (fig. 6A). Upon lipopolysaccharide stimulation, it increased to 1.07 ng/ml ($P < 0.0005$) and was attenuated by intratracheally applied ropivacaine by 59% ($P < 0.001$). Intravenous ropivacaine reduced CINC-1 by 69% ($P < 0.005$; fig. 6B).

As previously shown, MCP-1 plays a crucial role in neutrophil recruitment in the early endotoxin-induced lung injury and was therefore assessed in this study as well.[18] In control lungs, a MCP-1 concentration of 0.58 ng/ml was measured, which increased to 34.08 ng/ml in lipopolysaccharide-injured lungs ($P < 0.0005$; fig. 7A). In the presence of intratracheal ropivacaine, this increase was reduced to 17.02 ng/ml (51% down-regulation, $P < 0.01$). For intravenous intervention of ropivacaine, an 81% reduction was found ($P < 0.001$; fig. 7B).

Alveolocapillary Permeability

Albumin in the respiratory compartment is a reliable diagnostic parameter for the change of alveolocapillary permeability. To evaluate the effect of ropivacaine on vascular permeability, albumin content was determined in BALF. Intratracheally applied lipopolysaccharide caused an increase of albumin from 81 to 1,093 ng/ml ($P < 0.00005$; fig. 8A). In animals treated with ropivacaine intratracheally, a 46% attenuation of the albumin content was observed ($P < 0.0005$), whereas intravenous ropivacaine application decreased the albumin content by 38% ($P < 0.05$; fig. 8B).

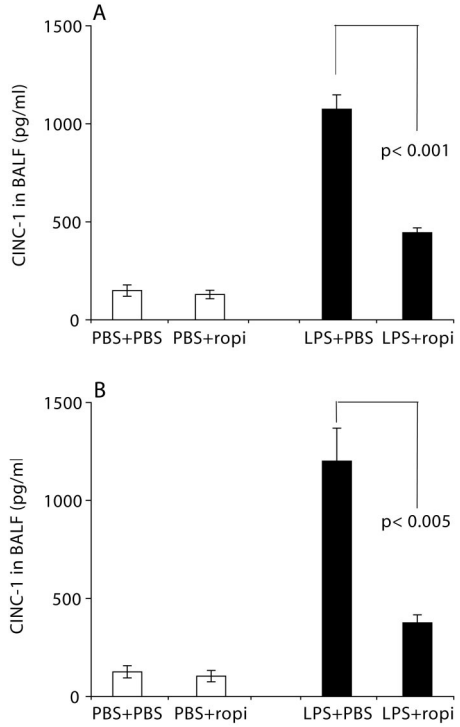

Fig. 6. (*A*) Cytokine-induced neutrophil chemoattractant 1 (CINC-1) protein determination in bronchoalveolar lavage fluid after intratracheal application of ropivacaine (ropi). Lipopolysaccharide (LPS), 150 μg, in 300 μl phosphate-buffered saline (PBS) was intratracheally instilled (or PBS alone for control animals) in the presence or absence of 1 mM ropivacaine. Bronchoalveolar lavage was performed 5 h later. CINC-1 was examined using a standard enzyme-linked immunosorbent assay. Values are mean ± SEM from five animals. (*B*) CINC-1 protein determination in bronchoalveolar lavage fluid after intravenous application of ropivacaine. Lipopolysaccharide, 150 μg, in 300 μl PBS was intratracheally instilled (or PBS alone for control animals). At the same time, 300 μl of 1 mM ropivacaine in PBS was intravenously applied (control animals with PBS only). Bronchoalveolar lavage was performed 5 h later. CINC-1 was examined using a standard enzyme-linked immunosorbent assay. Values are mean ± SEM from five animals.

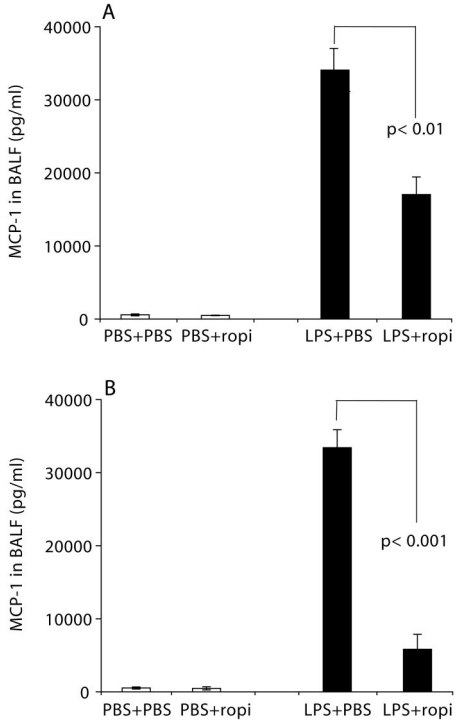

Fig. 7. (*A*) Monocyte-chemoattractant-protein 1 (MCP-1) protein determination bronchoalveolar lavage fluid (BALF) after intratracheal application of ropivacaine (ropi). Lipopolysaccharide (LPS), 150 μg, in 300 μl phosphate-buffered saline (PBS) was intratracheally instilled (or PBS alone for control animals) in the presence or absence of 1 mM ropivacaine. Bronchoalveolar lavage was performed 5 h later. MCP-1 was examined using a standard enzyme-linked immunosorbent assay. Values are mean ± SEM from five animals. (*B*) MCP-1 protein determination in BALF after intravenous application of ropivacaine. Lipopolysaccharide, 150 μg, in 300 μl PBS was intratracheally instilled (or PBS alone for control animals). At the same time, 300 μl of 1 mM ropivacaine in PBS was intravenously applied (control animals with PBS only). Bronchoalveolar lavage was performed 5 h later. MCP-1 was examined using a standard enzyme-linked immunosorbent assay. Values are mean ± SEM from five animals.

Discussion

In the current study, we have shown that ropivacaine is an effective antiinflammatory substance in a model of endotoxin-induced lung injury *in vitro* and *in vivo*. The use of *in vitro* ropivacaine significantly decreased ICAM-1–mediated neutrophil adherence and effector cell–induced killing in pulmonary endothelial and epithelial cells. In a model of ALI *in vivo*, ropivacaine showed effect on two important parameters of inflammation: It attenuated accumulation of effector cells, possibly by down-regulation of key chemotaxins, and in addition reduced alveolocapillary permeability. This diminished inflammatory response to lipopolysaccharide was observed not only for intravenous but also for intratracheal application of ropivacaine.

Lidocaine and other local anesthetics were recently shown to have various inhibitory effects in inflammatory processes. Most of these studies were performed in neutrophils, with focus on the expression of CD11b/CD18 or superoxide anion release.[19–21] All of these experiments aimed at the function of effector cells such as neutrophils and macrophages. Our goal was to determine the impact of ropivacaine, not on effector, but on target cells, which were exposed to noxious substances.

In a first approach, we tested the effect of ropivacaine on lipopolysaccharide-injured endothelial and epithelial lung cells *in vitro*. Because several studies have highlighted the importance of the respiratory epithelial compartment in the process of lung inflammation, we studied not only the vascular but also the respiratory compartment.[2,3,18,22] Ropivacaine suppressed both endothelial and epithelial ICAM-1 expression, with ICAM-1 being a key adhesion molecule at the beginning of the inflammatory reaction. Importantly, possible biologic consequences of decreased ICAM-1 expression, such as adhesion of effector cells and target cell killing, were diminished as well in our study. The antiinflammatory

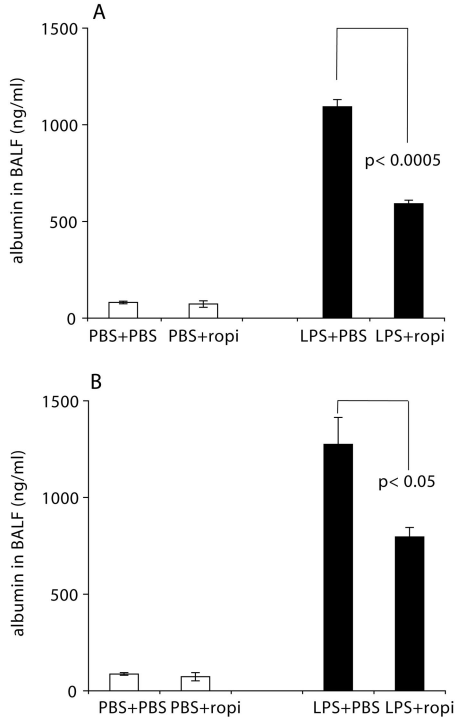

Fig. 8. (*A*) Albumin determination in bronchoalveolar lavage fluid (BALF) after intratracheal application of ropivacaine (ropi). Lipopolysaccharide (LPS), 150 μg, in 300 μl phosphate-buffered saline (PBS) was intratracheally instilled (or PBS alone for control animals) in the presence or absence of 1 mM ropivacaine. Bronchoalveolar lavage was performed 5 h later. Albumin was examined using a standard enzyme-linked immunosorbent assay. Values are mean ± SEM from five animals. (*B*) Albumin determination in BALF after intravenous application of ropivacaine. Lipopolysaccharide, 150 μg, in 300 μl PBS was intratracheally instilled (or PBS alone for control animals). At the same time, 300 μl of 1 mM ropivacaine in PBS was intravenously applied (control animals with PBS only). Bronchoalveolar lavage was performed 5 h later. Albumin was examined using a standard enzyme-linked immunosorbent assay. Values are mean ± SEM from five animals.

effect of local anesthetics on epithelial and endothelial cells described herein represents new information.

The effect of ropivacaine regarding mRNA expression for ICAM-1 represented a reverse concentration–response relation (fig. 1). Ropivacaine might interact with a membrane receptor, inducing an inhibitory cell-signaling pathway and thereby negatively interfering with the lipopolysaccharide-induced pathways such as toll-like receptors, CD14, or lipopolysaccharide-binding protein. With higher concentrations, ropivacaine molecules might change configuration, therefore not being able to interact anymore with the according membrane receptor. Regarding clinical applicability of ropivacaine, the phenomenon of its specific concentration–response curve would imply a certain positive aspect with regard to the margin of safety.

Another interesting phenomenon was the missing blocking effect of ropivacaine on cultured cells in the absence of lipopolysaccharide (fig. 1). This might give rise to the hypothesis that ropivacaine exclusively interferes with lipopolysaccharide-depending intracellular pathways.

Lidocaine has already been studied *in vivo* to determine a potential inhibitory effect of this local anesthetic substance in acute lung inflammation. Lidocaine significantly attenuated lung injury in a model of endotoxemia.[23–25] A similar protective effect with lidocaine was achieved in acid aspiration, reperfusion injury, and hyperoxia-induced lung injury.[26–28] However, none of these studies evaluated ropivacaine or an intratracheal application.

There are several reasons to consider and further investigate the use of the new local anesthetic ropivacaine in inflammatory lung processes because it might be safer. Although lidocaine has shown beneficial effects regarding the inflammation process in ALI, it was declared as a potentially harmful drug in patients with ALI.[29] In addition, some local anesthetics have been implicated as a cause of ALI.[30,31] It is known from lidocaine that inhibition of neutrophils also suppresses microbial clearance functions during infections. Other neutrophil-directed antiinflammatory agents, which have also been shown to inhibit ALI related to noninfectious causes, ultimately worsened the outcome in infection-related ALI.[32,33] Cardiotoxicity, as one of the most severe complications of local anesthetics, is another issue that raises concern when these drugs are used.[34,35] Although lidocaine has the longest history of being given systemically, there are also concerns, which have been defined. Lidocaine, when used to suppress ventricular fibrillation in acute myocardial infarction, has increased the incidence of asystolic arrest.[35] In addition, lidocaine might worsen congestive heart failure in patients with ALI.[34,36] Finally, ropivacaine has been shown to have a wider margin of safety as compared with lidocaine in experimental studies. Lidocaine, 0.6%, but not 0.1% ropivacaine, can induce apoptosis on a T-cell line, and higher neurotoxicity of lidocaine compared with ropivacaine was observed when tested on dorsal root ganglion neurons from chick embryos.[37,38]

Another interesting aspect of our study is the attenuation of ALI after intratracheal instillation of ropivacaine. The intratracheal approach essentially represents a local rather than a systemic administration, which would allow the potential effect to be targeted to the lungs with a possible reduction of undesired systemic effects. A study performed with lidocaine has shown that intravenous and intratracheal applications of lidocaine have the same effect regarding treatment of bronchial hyperactivity/constriction.[39] However, plasma concentration of lidocaine after intratracheally applied lidocaine treatment was only one third compared with systemic application. As direct instillation of endotoxin into lungs produces a characteristic pattern of inflammation similar to that found in acute respiratory distress syndrome, therapy directed toward limiting the local inflammatory response

would be an option for improving outcomes in humans with acute respiratory distress syndrome.[22]

In summary, we have shown that ropivacaine has strong antiinflammatory effects on neutrophils, but also on endothelial and epithelial lung cells *in vitro* and *in vivo*. Beside the topical effect of this local anesthetic, when intratracheally instilled, inflammatory actions were inhibited by low and clinically relevant doses of ropivacaine. It will be of further interest to elucidate the protective effects of ropivacaine *in vivo* to answer critical questions regarding the efficacy and safety of this new local anesthetic in the context of ALI.

The authors thank Irene Odermatt (Art Designer, Institute of Anesthesiology, University of Zurich, Zurich, Switzerland) and Christian Gasser (Art Designer, Institute of Physiology, University of Zurich) for development of illustrations.

References

1. Simon RH, Paine R 3rd: Participation of pulmonary alveolar epithelial cells in lung inflammation. J Lab Clin Med 1995; 126:108–18
2. Beck-Schimmer B, Schimmer RC, Warner RL, Schmal H, Nordblom G, Flory CM, Lesch ME, Friedl HP, Schrier DJ, Ward PA: Expression of lung vascular and airway ICAM-1 after exposure to bacterial lipopolysaccharide. Am J Respir Cell Mol Biol 1997; 17:344–52
3. Beck-Schimmer B, Madjdpour C, Kneller S, Ziegler U, Pasch T, Wuethrich RP, Ward PA, Schimmer RC: Role of alveolar epithelial ICAM-1 in lipopolysaccharide-induced lung inflammation. Eur Resp J 2002; 19:1142–50
4. Paine R III, Rolfe MW, Standiford TJ, Burdick MD, Rollins BJ, Strieter RM: MCP-1 expression by rat type II alveolar epithelial cells in primary culture. J Immunol 1993; 150:4561–70
5. Barton WW, Wilcoxen S, Christensen PJ, Paine R: Disparate cytokine regulation of ICAM-1 in rat alveolar epithelial cells and pulmonary endothelial cells *in vitro*. Am J Physiol 1995; 269:L127–35
6. Beck-Schimmer B, Schimmer RC, Schmal H, Flory CM, Friedl HP, Pasch T, Ward PA: Characterization of rat lung ICAM-1. Inflamm Res 1998; 47:308–15
7. Nishina K, Zhang F, Nielsen LD, Edeen K, Wang J, Mason RJ: Expression of CINC-2 beta is related to the state of differentiation of alveolar epithelial cells. Am J Respir Cell Mol Biol 2005; 33:505–12
8. Peck SL, Johnston RB Jr, Horwitz LD: Reduced neutrophil superoxide anion release after prolonged infusions of lidocaine. J Pharmacol Exp Ther 1985; 235:418–22
9. Cullen BF, Haschke RH: Local anesthetic inhibition of phagocytosis and metabolism of human leukocytes. ANESTHESIOLOGY 1974; 40:142–6
10. Scott DB, Lee A, Fagan D, Bowler GM, Bloomfield P, Lundh R: Acute toxicity of ropivacaine compared with that of bupivacaine. Anesth Analg 1989; 69:563–9
11. Santos AC, Arthur GR, Wlody D, De Armas P, Morishima HO, Finster M: Comparative systemic toxicity of ropivacaine and bupivacaine in nonpregnant and pregnant ewes. ANESTHESIOLOGY 1995; 82:734–40
12. Knudsen K, Beckman Suurkula M, Blomberg S, Sjovall J, Edvardsson N: Central nervous and cardiovascular effects of i.v. infusions of ropivacaine, bupivacaine and placebo in volunteers. Br J Anaesth 1997; 78:507–14
13. Arlander E, Ost A, Stahlberg D, Lofberg R: Ropivacaine gel in active distal ulcerative colitis and proctitis: A pharmacokinetic and exploratory clinical study. Aliment Pharmacol Ther 1996; 10:73–81
14. Douglas WH, Kaighn ME: Clonal isolation of differentiated rat lung cells. In Vitro 1974; 10:230–7
15. Madjdpour C, Jewell UR, Kneller S, Ziegler U, Schwendener R, Booy C, Klausli L, Pasch T, Schimmer RC, Beck-Schimmer B: Decreased alveolar oxygen induces lung inflammation. Am J Physiol Lung Cell Mol Physiol 2003; 284:L360–7
16. Madjdpour C, Oertli B, Ziegler U, Bonvini JM, Pasch T, Beck-Schimmer B: Lipopolysaccharide induces functional ICAM-1 expression in rat alveolar epithelial cells *in vitro*. Am J Physiol Lung Cell Mol Physiol 2000; 278:L572–9
17. Nemzek JA, Call DR, Ebong SJ, Newcomb DE, Bolgos GL, Remick DG: Immunopathology of a two-hit murine model of acid aspiration lung injury. Am J Physiol Lung Cell Mol Physiol 2000; 278:L512–20
18. Beck-Schimmer B, Schwendener R, Pasch T, Reyes L, Booy C, Schimmer RC: Alveolar macrophages regulate neutrophil recruitment in endotoxin-induced lung injury. Respir Res 2005; 6:61–74
19. Sasagawa S: Inhibitory effects of local anesthetics on migration, extracellular release of lysosomal enzyme, and superoxide anion production in human polymorphonuclear leukocytes. Immunopharmacol Immunotoxicol 1991; 13:607–22
20. Ohsaka A, Saionji K, Sato N, Igari J: Local anesthetic lidocaine inhibits the effect of granulocyte colony-stimulating factor on human neutrophil functions. Exp Hematol 1994; 22:460–6
21. Azuma Y, Shinohara M, Wang PL, Suese Y, Yasuda H, Ohura K: Comparison of inhibitory effects of local anesthetics on immune functions of neutrophils. Int J Immunopharmacol 2000; 22:789–96
22. Blackwell TS, Christman JW: Defining the lung's response to endotoxin. Am J Respir Crit Care Med 2001; 163:1516–7
23. Taniguchi T, Shibata K, Yamamoto K, Mizukoshi Y, Kobayashi T: Effects of lidocaine administration on hemodynamics and cytokine responses to endotoxemia in rabbits. Crit Care Med 2000; 28:755–9
24. Mikawa K, Maekawa N, Nishina K, Takao Y, Yaku H, Obara H: Effect of lidocaine pretreatment on endotoxin-induced lung injury in rabbits. ANESTHESIOLOGY 1994; 81:689–99
25. Schmidt W, Schmidt H, Bauer H, Gebhard MM, Martin E: Influence of lidocaine on endotoxin-induced leukocyte–endothelial cell adhesion and macromolecular leakage *in vivo*. ANESTHESIOLOGY 1997; 87:617–24
26. Nishina K, Mikawa K, Takao Y, Shiga M, Maekawa N, Obara H: Intravenous lidocaine attenuates acute lung injury induced by hydrochloric acid aspiration in rabbits. ANESTHESIOLOGY 1998; 88:1300–9
27. Schmid RA, Yamashita M, Ando K, Tanaka Y, Cooper JD, Patterson GA: Lidocaine reduces reperfusion injury and neutrophil migration in canine lung allografts. Ann Thorac Surg 1996; 61:949–55
28. Takao Y, Mikawa K, Nishina K, Maekawa N, Obara H: Lidocaine attenuates hyperoxic lung injury in rabbits. Acta Anaesthesiol Scand 1996; 40:318–25
29. DePietro MR, Eichacker PQ: Lidocaine for acute lung injury: Questions still to answer. Crit Care Med 2000; 28:589–91
30. Promisloff RA, DuPont DC: Death from ARDS and cardiovascular collapse following lidocaine administration (communications to the editor). Chest 1983; 83:585
31. Howard JJ, Mohsenifar Z, Simons SM: Adult respiratory distress syndrome following administration of lidocaine. Chest 1982; 81:644–5
32. Eichacker PQ, Farese A, Hoffman WD, Banks SM, Mouginis T, Richmond S, Kuo GC, Macvittie TJ, Natanson C: Leukocyte CD11b/18 antigen-directed monoclonal antibody improves early survival and decreases hypoxemia in dogs challenged with tumor necrosis factor. Am Rev Respir Dis 1992; 145:1023–9
33. Freeman BD, Correa R, Karzai W, Natanson C, Patterson M, Banks S, Fitz Y, Danner RL, Wilson L, Eichacker PQ: Controlled trials of rG-CSF and CD11b-directed MAb during hyperoxia and *E. coli* pneumonia in rats. J Appl Physiol 1996; 80:2066–76
34. Pharand C, Kluger J, O'Rangers E, Ujhelyi M, Fisher J, Chow M: Lidocaine prophylaxis for fatal ventricular arrhythmias after acute myocardial infarction. Clin Pharmacol Ther 1995; 57:471–8
35. Gheorghiade M, Ruzumna P, Borzak S, Havstad S, Ali A, Goldstein S: Decline in the rate of hospital mortality from acute myocardial infarction: Impact of changing management strategies. Am Heart J 1996; 131:250–6
36. Ballas SL, Baughman KL, Griffith LS, Veltri EP: Mexiletine-associated left ventricular dysfunction: A case study. Md Med J 1991; 40:519–20
37. Boselli E, Duflo F, Debon R, Allaouchiche B, Chassard D, Thomas L, Portoukalian J: The induction of apoptosis by local anesthetics: A comparison between lidocaine and ropivacaine. Anesth Analg 2003; 96:755–6
38. Radwan IA, Saito S, Goto F: The neurotoxicity of local anesthetics on growing neurons: A comparative study of lidocaine, bupivacaine, mepivacaine, and ropivacaine. Anesth Analg 2002; 94:319–24
39. Groeben H, Silvanus MT, Beste M, Peters J: Both intravenous and inhaled lidocaine attenuate reflex bronchoconstriction but at different plasma concentrations. Am J Respir Crit Care Med 1999; 159:530–5