Reactive Nitrogen Species Inhibit Alveolar Epithelial Fluid Transport After Hemorrhagic Shock in Rats

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Reactive Nitrogen Species Inhibit Alveolar Epithelial Fluid Transport After Hemorrhagic Shock in Rats

Jean-François Pittet, Le N. Lu, David G. Morris, Kathrin Modelska, William J. Welch, Hannah V. Carey, Jeremie Roux, and Michael A. Matthay

Our recent experimental work demonstrated that a neutrophil-dependent inflammatory response in the lung prevented the normal up-regulation of alveolar fluid clearance by catecholamines following hemorrhagic shock. In this study, we tested the hypothesis that the release of NO within the airspaces of the lung was responsible for the shock-mediated failure of the alveolar epithelium to respond to catecholamines in rats. Hemorrhagic shock was associated with an inducible NO synthase (iNOS)-dependent increase in the lung production of NO and a failure of the alveolar epithelium to up-regulate vectorial fluid transport in response to â-adrenergic agonists. Inhibition of iNOS restored the normal catecholamine-mediated up-regulation of alveolar liquid clearance. Airspace instillation of dibutyryl cAMP, a stable analog of cAMP, restored the normal fluid transport capacity of the alveolar epithelium after prolonged hemorrhagic shock, whereas direct stimulation of adenyl cyclase by forskolin had no effect. Pretreatment with pyrrolidine dithiocarbamate or sulfasalazine attenuated the iNOS-dependent production of NO in the lung and restored the normal up-regulation of alveolar fluid clearance by catecholamines after prolonged hemorrhagic shock. Based on in vitro studies with an alveolar epithelial cell line, A549 cells, the effect of sulfasalazine appeared to be mediated in part by inhibition of IκB protein degradation. In summary, these results provide the first in vivo evidence that NO, released within the airspaces of the lung probably secondary to the NF-κB-dependent activation of iNOS, is a major proximal inflammatory mediator that limits the rate of alveolar epithelial transport after prolonged hemorrhagic shock by directly impairing the function of membrane proteins involved in the â-adrenergic receptor-cAMP signaling pathway in alveolar epithelium. The Journal of Immunology, 2001, 166: 6301–6310.

Major trauma associated with hemorrhagic shock is one of the most common and important causes of acute lung injury and pulmonary edema in patients. Hemorrhagic shock initiates an inflammatory response in the lung characterized by up-regulation of pro-inflammatory cytokines (2–4) and accumulation of neutrophils (5) that contribute to the organ damage after shock. For example, normal up-regulation of alveolar fluid clearance by endogenous catecholamines released during septic or hemorrhagic shock (6–8) is inhibited by a neutrophil-dependent oxidative injury to the alveolar epithelium after prolonged hemorrhagic shock (9). The mechanisms by which hemorrhage triggers this inflammatory response in the lung include heightened â-adrenergic activity and release of oxidant radicals and IL-1β in the airspaces (9–11), mostly by neutrophils that accumulate in the lung after the onset of hemorrhagic shock (9, 12).

NO is one of the important oxidant radicals released during hemorrhagic shock in lung and liver by the activation of inducible NO synthase (iNOS). NO plays an essential role in initiating the inflammatory response in these organs after onset of hemorrhage (13–15). Moreover, the inhibition of iNOS by pretreatment with N-(iminomethyl)-l-lysine (l-NIL) in hemorrhaged rats was associated with a significant decrease in the shock-dependent neutrophil infiltration in the lung and with a significant reduction in the shock-mediated increase in extravascular lung water (13). However, it is still unknown whether induced NO could affect the â-adrenergic receptor-cAMP signaling pathway of the alveolar epithelium in vivo and thus be a proximal mediator responsible for the oxidant-mediated inhibition of alveolar epithelial fluid transport after prolonged hemorrhagic shock.

Therefore, the first objective was to determine whether the release of NO in the airspaces of the lung was responsible for the shock-mediated failure of the alveolar epithelium to respond to catecholamines. Hemorrhagic shock was associated with a significant increase in the production of nitrite secondary to the expression of iNOS in the lung and a failure of the alveolar epithelium to up-regulate vectorial fluid transport in response to â-adrenergic agonists. Inhibition of iNOS by two specific iNOS inhibitors restored the normal alveolar fluid transport capacity after hemorrhagic shock. The second objective was to determine whether the shock-mediated release of NO within the airspaces not only affects the function of the â-adrenergic receptor, but also other membrane proteins, such as adenyl cyclase, that are involved in the â-adrenergic receptor-cAMP signaling pathway in alveolar epithelium.

The third objective was to test the hypothesis that the activation of the transcription factor, NF-κB, may be responsible for the release of NO in the airspaces of the lung.

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2 This work was primarily supported by National Heart, Lung and Blood Institute Grant HL 51854. This project has also been supported in part through award of a research grant from the American Lung Association (to K.M.) and a Fellowship Grant HL 51854. This project has also been supported in part through award of a research grant from the American Lung Association California (to J.F.P.) and a Fellowship Grant HL 51854. This project has also been supported in part through award of a research grant from the American Lung Association California (to K.M.).

3 Abbreviations used in this paper iNOS, inducible NO synthase; l-NIL, N-(iminomethyl)-l-lysine; dc-AMP, dibutyryl cAMP; PDTC, pyrrolidine dithiocarbamate.
Materials and Methods

The protocol for these studies was approved by the University of California at San Francisco Animal Research Committee.

Lung barrier function studies

Surgical preparation and ventilation. Male Sprague Dawley rats weighing 300–350 g were anesthetized with pentobarbital (60 mg/kg i.p.), and anesthesia was maintained with 30 mg/kg of pentobarbital sodium every 2 h. An endotracheal tube (PE 220) was inserted through a tracheotomy. Pancuronium bromide (0.3 mg/kg i.v.) was given for neuromuscular blockade. Catheters (PE 50) were inserted into both carotid arteries to monitor systemic arterial pressure, obtain blood samples, and withdraw blood for induction of prolonged hemorrhagic shock. A catheter was also inserted into the jugular vein to monitor central venous pressure. The rats were maintained in the left lateral decubitus position during the experiments and were ventilated with a constant-volume pump (Harvard Apparatus, Holliston, MA) with an inspired oxygen fraction of 1.0, peak airway pressures of 8–12 cm H₂O, supplemented with positive end-expiratory pressure of 3 cm H₂O. The respiratory rate was adjusted to maintain the PaCO₂ between 35 and 40 mm Hg during the baseline period.

Preparation of instillate. A 5% bovine albumin solution was prepared using Ringer’s lactate and adjusted with NaCl to be iso-osmolar with the circulating plasma of the rat, as previously published (8, 9). Anhydrous Evan’s blue dye (0.5 mg) was added to the albumin solution to confirm the location of the instillate at the end of the study, and 1 μCi of 125I-labeled human serum albumin (Frost Laboratories, Quebec, Canada) was also added to the albumin solution. The 125I-labeled albumin served as the alveolar protein tracer in all experiments. A sample of the instilled solution was saved for total protein measurement, radioactivity counts, and water-to-dry weight ratio measurements so that the dry weight of the protein solution could be subtracted from the final lung water calculation. In some experiments, propranolol, dibutyryl cAMP (dc-AMP), or forskolin was added to the instilled protein solution.

General protocol

In all experiments, after the surgery, heart rate and systemic blood pressure were allowed to stabilize for 60 min (Fig. 1, A–C). The rat was placed in the left lateral decubitus position to facilitate liquid deposition into the left lung 300 min after the onset of hemorrhagic shock. Hemorrhagic shock was induced by withdrawing blood from the carotid artery to maintain a mean systemic arterial pressure of 30–35 mm Hg for 60 min. This corresponded to the removal of 9–12 ml of blood. After 1 h of hemorrhagic shock the rats were resuscitated with intravascular 4% albumin solution in 0.9% NaCl over 30 min to maintain a central venous pressure <8 mm Hg, as we have done before (8, 9). The volume of 4% albumin solution administered was twice the amount of blood withdrawn.

A vascular tracer, 1 μCi of 125I-labeled human albumin, was injected into the blood 270 min after the onset of hemorrhagic shock to calculate the flux of plasma protein into the lung interstitium, as previously published (8, 9). An alveolar tracer, 1 μCi of 125I-labeled albumin in 3 ml/kg of the 5% bovine albumin solution, was instilled into the left lower lobe 30 min later to calculate the flux of protein from the airspaces into the circulating plasma. At the end of the experiment (1 h after the beginning of the alveolar instillation), the abdomen was opened and the rats were exsanguinated by transecting the abdominal aorta. Urine was obtained for radioactivity counts. The lungs were removed through a median sternotomy. An alveolar protein tracer in all experiments. A sample of the instilled solution containing 10⁻³⁸ M epinephrine for the last hour of the experiment (n = 3).

Group 2. Inhibition of iNOS with amino guanidine or l-NIL (n = 23). NO is one of the important oxidant radicals released during hemorrhagic shock and plays an essential role in the initiation of the inflammatory response in the lung (13). Thus, experiments were performed to determine whether the production of NO in the airspaces would affect alveolar fluid clearance after hemorrhagic shock and fluid resuscitation. Rats were pretreated with either amino guanidine (i.v. bolus 180 mg/kg, then an i.v. infusion of 1.04 mg/kg/h) (n = 4) or l-NIL (4 mg/kg i.p. repeated every 2 h) (n = 5), two unrelated iNOS inhibitors. Epinephrine (10⁻⁷ M) was added to the solution instilled into the distal airspaces of the lung of hemorrhaged rats. Control studies included rats that were not hemorrhaged or fluid resuscitated, but total airspaces instilled with an albumin solution containing 10⁻³⁸ M epinephrine for the last hour of the experiment (n = 3).

Group 3. Stimulation with dc-AMP or forskolin (n = 14). These experiments were designed to determine whether NO would not only affect the function of the β-adrenergic receptor, but also that of other membrane receptors, such as adenylyl cyclase, involved in the β-adrenergic receptor-cAMP signaling pathway. Thus, forskolin (5 × 10⁻⁴ M) (Sigma, St. Louis, MO), an adenylyl cyclase agonist, was added to the protein solution instilled into the distal airspaces (n = 3). In addition, the instilled protein solution contained propranolol at a concentration (10⁻⁴ M) that inhibits the β-adrenergic receptors on the alveolar epithelial cells (7), and aminophylline, a phosphodiesterase inhibitor, to prevent the rapid degradation of cAMP (10⁻⁶ M). Aminophylline was also given as continuous i.v. infusion at the

![FIGURE 1. A, B, and C. General experimental protocol (see Materials and Methods for further explanation).](http://www.jimmunol.org/content/151/6/6302/F1.large.jpg)
rate of 1 mg/kg/h that was started 30 min before onset of hemorrhagic shock. As a positive control, a second series of hemorrhaged rats had their airspaces instilled with dc-AMP (10−5 M; Sigma), a stable analog of cAMP (n = 4). These rats also had their airspaces instilled with propranolol (10−5 M) and were given aminophylline (10−4 M) as a continuous i.v. infusion (1 mg/kg/h).

Control studies included rats that were neither hemorrhaged nor fluid resuscitated, but had their distal airspaces instilled with an albumin solution containing forskolin (5 × 10−5 M) (n = 3) or dc-AMP (10−5 M) (n = 4). In addition, the instilled protein solution contained propranolol (10−5 M) and aminophylline (10−4 M). Aminophylline was also given as continuous i.v. infusion at the rate of 1 mg/kg/h throughout the experiment.

**Group 4. Inhibition of NF-κB activation by pyrroldione dithiocarbamate (PDTC) or sulfasalazine (n = 21).** Activation of the proinflammatory transcriptional factor, NF-κB, occurs early (15 min) after the onset of septic (16) and hemorrhagic shock (17). Also, the in vivo inhibition of NF-κB activation prevents endotoxin-dependent iNOS expression in the lung (18). Thus, experiments were performed to determine whether inhibition of activation of NF-κB in the lung would affect the release of NO within the airspaces of the lung after prolonged hemorrhagic shock. Inhibition of NF-κB activation was achieved by adding sulfasalazine (130 mg/kg/day) (n = 4) to the drinking water for 3 wk or by pretreating rats with PDTC (200 mg/kg i.p.) (n = 4) 30 min before onset of hemorrhagic shock. Sulfasalazine has been shown recently to be a potent and specific inhibitor of NF-κB activation by TNF-α (19). LPS-induced macrophages secrete p38 mitogen-activated protein (MAP) kinases, and p38 protein expression is inhibited by sulfasalazine. Inhibition of NF-κB would be expected to have a significant effect on NO production. These rats also had their airspaces instilled with propranolol (10−3 M). Aminophylline (10−4 M) was added to the protein solution instilled into the distal airspaces of the lung of hemorrhaged rats. Control studies included rats that were not hemorrhaged or fluid resuscitated, but were pretreated with either sulfasalazine (130 mg/kg/day) (n = 3) to the drinking water for 3 wk or with PDTC (200 mg/kg i.p.) (n = 4) and were instilled with a protein solution containing epinephrine (10−4 M). In pilot experiments (n = 6), neither sulfasalazine nor PDTC affected alveolar fluid clearance in control rats.

**Measurements**

**Hemodynamics, pulmonary gas exchange, and protein concentration.** Systemic arterial, central venous, and airway pressures were continuously measured. Arterial blood gases were measured at 1-h intervals. Samples from the instilled protein solution, from final distal airspace fluid, and from initial and final blood were collected to measure total protein concentration with an automated analyzer (AA2; Technicon, Tarrytown, NY).

**Albumin flux across endothelial and epithelial barriers.** Two different methods were used to measure the flux of albumin across the lung endothelial and epithelial barriers, as we have done before (7–9). The first method measures residual 125I-labeled albumin (the airspace protein tracer) in the lungs as well as accumulation of 125I-labeled albumin in plasma. The second method measures 35S-labeled albumin (the vascular protein tracer) in the extravascular space of the lungs (7–9).

**Alveolar fluid clearance.** Changes in the concentration of the nonlabeled bovine albumin and the instilled 125I-labeled albumin over the study period (1 h) were used to measure fluid clearance from the distal airspaces, as we have done before (7–9). There is a good correlation between the changes in the concentration of instilled nonlabeled bovine albumin and 125I-labeled albumin. Because some reabsorption may have occurred across distal bronchial epithelium, the term alveolar does not imply that all fluid reabsorption occurred at the alveolar level.

**Tracer binding measurement.** The 125I binding to albumin was measured by adding trichloroacetic acid (20%) to plasma and alveolar fluid samples. None of the fluid samples ever had >1% of unbound iodine present.

**Production of nitrite by alveolar macrophages**

To determine nitrite production by alveolar macrophages after prolonged shock, a first series of experiments included rats (n = 4) that were hemorrhaged and fluid resuscitated as described in General Protocol. Then, the rats were exsanguinated, and their lungs were inflated to total lung capacity with cold sterile PBS (10 ml) and lavaged twice. A second series of experiments included rats that were pretreated with t-NIL (4 mg/kg i.p. repeated every 2 h) (n = 4) before they underwent prolonged hemorrhagic shock and fluid resuscitation as described previously. At the end of the experiments, the lungs were inflated to total lung capacity with cold sterile PBS (10 ml) and lavaged twice.

A third series of experiments included rats that were pretreated with sulfasalazine (130 mg/kg/day added to the drinking water for 3 wk) (n = 4) before they underwent prolonged hemorrhagic shock and fluid resuscitation as described previously. At the end of the experiments, the lungs were inflated to total lung capacity with cold sterile PBS (10 ml) and lavaged twice.

Control studies included 1) rats (n = 4) that were neither hemorrhaged nor fluid resuscitated and had their airspaces lavaged twice at the end of the studies with cold sterile PBS (10 ml); and 2) rats that were neither hemorrhaged nor fluid resuscitated, but were either pretreated with t-NIL (4 mg/kg i.p. repeated every 2 h) (n = 4) or sulfasalazine (130 mg/kg/day added to the drinking water for 3 wk) (n = 4) and had their airspaces lavaged twice at the end of the studies with cold sterile PBS (10 ml). In all experiments, the lavage samples were then centrifuged at 800 × g for 10 min at 4°C to remove cells. The cell pellet was resuspended in 1 ml of sterile RBC lysis buffer (Sigma) for 10 min at room temperature. The cells were then washed and resuspended in saline and cytopsin and cell differential staining (Diff-Quick; Dade Diagnostics, Aguada, PR). Cell concentration was adjusted by dilution in serum-free RPMI 1640 medium (University of California at San Francisco Cell Culture Facility) to 5 × 106 cells/ml and equal number of cells (1 × 105) were plated on 96-well tissue culture plates (Falcon, Lincoln Park, NJ). The cells were treated with a range of LPS concentrations from 0 to 10 ng/ml (Escherichia coli 055:B5; Sigma). The alveolar macrophages were then incubated at 37°C (5% CO2) for 24 h, and cell supernatants were collected.

Nitrite production was quantified in the harvested supernatant using a modified Griess Reagent (Sigma) with measurement of visible light absorption at 540 nm. Cell differential counts were performed manually. Results are expressed as nitrite production (μM) over 24 h × 106 cells.

**Western blot measurement for iNOS**

Frozen lungs from hemorrhaged and control rats (n = 5 in each group) were thawed in a tissue homogenization buffer (100 mg lung tissue/ml buffer) including 50 mM Tris, pH 7.4, 20 mM NaCl, 10 mM KCl, 0.1 mM DTT, 1 mM EDTA, 1% SDS, and a protease inhibitor mixture. Then, the lung tissue was homogenized with a polytron (3 × 15-s bursts) and the samples were immediately boiled for 5 min at 95–100°C. Samples were sonicated 2 × 15 s at room temperature. Then the samples were centrifuged at 14,000 × g at 4°C for 30 min and the supernatant was quickly frozen at −70°C. The protein concentration was measured using the bicinchoninic acid kit assay with BSA as the standard (Pierce, Rockford, IL). A portion of the lung tissue lysate was then boiled in Laemmli sample buffer for 5 min at 95–100°C before loading 25 μg of total protein per lane on a 10% Tris-glycine SDS-polyacylamide gel. The electrophoretically separated proteins were subsequently transferred to nitrocellulose membranes, which were blocked with 5% milk protein in PBS for 120 min. To detect iNOS protein, nitrocellulose membranes were immunoblotted using a commercially available 1:500 or 1:1000 dilution of a monoclonal anti-mouse iNOS Ab overnight at 4°C (Transduction Laboratories, Lexington, KY). The blot was then incubated with HRP-labeled goat anti-mouse Ig (dilution 1:2000). Protein bands were visualized using a chemiluminescence method (SuperSignal; Pierce) and quantitated using a digital image analysis system (Chemilimager; Alpha Innotech, San Leandro, CA).

**A549 cell experiments**

**Cell culture.** All experiments involved A549 cells (American Type Culture Collection, Manassas, VA), a human lung adenocarcinoma cell line representative of distal respiratory epithelium. These cells have previously been shown to be a useful model for studying in vitro NF-κB regulation in response to proinflammatory stimuli (20). Cells were maintained in a room air/5% CO2 incubator at 37°C using DMEM (Life Technologies, Grand Island, NY) containing 10% FBS and penicillin/streptomycin (Life Technologies).

**Transient transfection and luciferase assay.** To measure NF-κB regulation in response to proinflammatory stimuli, A549 cells were transiently transfected with a plasmid in which the luciferase gene was driven by three tandem NF-κB binding motifs followed by a minimal IFN-γ promoter (three binding sites for NF-κB-Luc, a gift of H. Wong, University of Cincinnati, Cincinnati, OH). This plasmid previously was demonstrated to be a sensitive tool to specifically evaluate NF-κB activation (20).

Cells were transfected in triplicate, in six-well plates, at a density of 300,000 cells per well by incubation with cationic liposomes (Fugene;
Roche, Indianapolis, IN) for 48 h in DMEM. Then, the cells were exposed to a mixture of proinflammatory cytokines (TNF-α, IL-1β, IFN-γ; Boehringer Mannheim, Indianapolis, IN) or its vehicle at a concentration of 10 ng/ml. A group of cells were pretreated with sulfasalazine (2 mM) 3 h before exposure to proinflammatory cytokines. Twenty-four hours later, cellular proteins were extracted and analyzed for luciferase activity according to the manufacturer’s instruction (Promega, Madison, WI) using a luminometer. Luciferase activity was corrected for total cellular protein and reported as fold-induction over the control cells (cells that were transfected and treated with cytokine vehicle alone).

**EMSA for NF-κB and Western blot measurement for IκBα.** A549 cells were plated in 100-mm culture dishes at a density of 1,000,000 cells per dish and exposed 24 h later to a mixture of proinflammatory cytokines (TNF-α, IL-1β, IFN-γ; Boehringer Mannheim) or its vehicle for 12 min. Then, nuclear protein extraction was performed on ice with ice-cold radi- cal. Cells were initially washed with cold PBS and harvested by scraping. The cells were pelleted in 1 ml of PBS at 5000 × g at 4°C for 10 min. The pellets were washed twice with PBS and resuspended in lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.4% Nonidet P-40, and 0.5 mM PMSF). The suspension was incubated on ice for 15 min and then centrifuged (10,000 × g at 4°C for 10 min. The supernatant (cytoplasmic proteins) was saved for Western blot analysis, and 1 cell pellet volume of extraction buffer was added (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.5 mM DTT, 1 mM EDTA, 0.5 mM PMSF). The suspension was incubated on ice for 5 min and then centrifuged at 16,000 × g at 4°C for 30 min. The supernatant (nuclear proteins) was collected and kept at −70°C until use. The protein concentration was determined using the bicinchoninic acid assay kit with BSA as the standard (Pierce).

EMSA was performed using a NF-κB consensus nucleotide probe (5’-ATG TGA GGC GAC TTT CCC AGG C-3’) that was end-labeled with [γ-32P]ATP (Amersham Life Science, Arlington Heights, IL). Nuclear protein (5 μg) was incubated with 100,000 cpm of 32P-labeled NF-κB consensus nucleotide for 20 min in a total volume of 16.5 μl in a binding buffer consisting of 10 mM Tris buffer, pH 7.5, 1 mM MgCl2, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 4% glycerol, and 1 μg of poly(dI-dC) (Phar- macia, Piscataway, NJ). The specificity of the DNA/protein binding was determined by competition reactions in which a 100-fold molar excess of unlabeled NF-κB oligonucleotide was added to the reaction. After incubation, the samples were loaded onto a nondenaturing minigel (5% Tris-glycine 0.1% SDS), and electrophoresis was performed at 4°C for 10 min. Each gel was then dried and was subjected to autoradiography.

To detect IκBα protein by Western blotting, samples of cytoplasmic proteins saved during the extraction of nuclear proteins were boiled in Laemmli sample buffer for 5 min at 95–100°C before loading 25 μg of total protein per lane on a 10% Tris-glycine SDS-polyacrylamide gel. The elec- trophorically separated proteins were subsequently transferred to nitrocellulose membranes, which were blocked with 5% milk protein in PBS for 120 min. Then, nitrocellulose membranes were immunoblotted using a 1:2500 dilution of a polyclonal anti-rabbit IgG (Sigma, St. Louis, MO) for 48 h in DMEM. Then, the cells were exposed to a mixture of proinflammatory cytokines (TNF-α, IL-1β, IFN-γ; Boehringer Mannheim) or its vehicle for 12 min. Then, nuclear protein extraction was performed on ice with ice-cold radi- cal. Cells were initially washed with cold PBS and harvested by scraping. The cells were pelleted in 1 ml of PBS at 5000 × g at 4°C for 10 min. The pellets were washed twice with PBS and resuspended in lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.4% Nonidet P-40, and 0.5 mM PMSF). The suspension was incubated on ice for 15 min and then centrifuged at 16,000 × g at 4°C for 30 min. The supernatant (nuclear proteins) was collected and kept at −70°C until use. The protein concentration was determined using the bicinchoninic acid assay kit with BSA as the standard (Pierce).

The blot was then incubated with HRP-labeled goat anti-rabbit IgG (dilution 1:1500). Protein bands were visualized using a chemilumines- cence method (SuperSignal; Pierce) and quantitated using a digital image analysis system (Chemilumager; Alpha Innotech).

**Results**

**Hemorrhagic shock decreases alveolar epithelial fluid transport**

To induce hemorrhagic shock, 38 ± 3% of the blood volume was withdrawn (9.5 ± 0.7 ml of blood) causing systemic arterial hypotension (34 ± 5 vs 104 ± 4 mm Hg, p < 0.05) and metabolic acidosis (arterial pH: 7.22 ± 0.02 vs 7.42 ± 0.01, p < 0.05) with a calculated base deficit of the arterial blood of −15.8 ± 2.3 in hemorrhaged rats vs −0.5 ± 0.4 in controls (p < 0.05) at the end of the ischemic phase of shock (Fig. 1).

Hemorrhagic shock was associated with a failure of the alveolar epithelium to increase vectorial fluid transport in response to cat- echolamines, despite the absence of any increase in the alveolar epithelial permeability to protein. Protein flux (125I-labeled albu- min) from the airspaces to the plasma was comparable in hemor- rhaged and control rats (0.6 ± 0.2 vs 0.4 ± 0.2, NS). The final- to-initial distal airspace unlabeled protein concentration did not increase in hemorrhaged rats despite adding epinephrine to the instilled protein solution to maximize the response of β-adrenergic receptors (Table I). In contrast, when epinephrine was added to the protein solution instilled into the alveolar space of control non- hemorrhaged rats, the expected increase in airspace unlabeled protein concentration occurred (Table I). In response to cat- echolamines, alveolar fluid clearance significantly increased in control rats, but not in hemorrhaged rats (Fig. 2). Comparable val- ues were obtained when alveolar fluid clearance was measured with the 125I-labeled albumin (control rats: 32 ± 2 to 42 ± 2%, p < 0.05; hemorrhaged rats: 33 ± 2 to 31 ± 3%, NS). Finally, hemorrhagic shock was associated with a small increase in lung

Table I. **Effect of iNOS or NF-κB inhibition on change in alveolar fluid protein concentration after severe hemorrhagic shock**

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>n</th>
<th>Initial</th>
<th>Final</th>
<th>F/I Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls – no shock</td>
<td>9</td>
<td>4.70 ± 0.14</td>
<td>6.40 ± 0.22</td>
<td>1.34 ± 0.06</td>
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<tr>
<td>Controls + al. epinephrine</td>
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<td>4.45 ± 0.36</td>
<td>7.10 ± 0.33</td>
<td>1.61 ± 0.08*</td>
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<td>Controls + al. epinephrine and amino guanidine</td>
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<td>4.45 ± 0.16</td>
<td>6.97 ± 0.19</td>
<td>1.57 ± 0.03*</td>
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<tr>
<td>Controls + al. epinephrine and L-NIL</td>
<td>4</td>
<td>4.65 ± 0.08</td>
<td>7.26 ± 0.20</td>
<td>1.56 ± 0.02*</td>
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<td>Controls + al. epinephrine and PDTC</td>
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<td>4.36 ± 0.09</td>
<td>6.51 ± 0.05</td>
<td>1.49 ± 0.03*</td>
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<tr>
<td>Controls + al. epinephrine and sulfasalazine</td>
<td>3</td>
<td>4.27 ± 0.17</td>
<td>6.53 ± 0.25</td>
<td>1.53 ± 0.01*</td>
</tr>
<tr>
<td>Hemorrhagic shock</td>
<td>6</td>
<td>4.47 ± 0.08</td>
<td>6.01 ± 0.16</td>
<td>1.34 ± 0.02</td>
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<td>4.62 ± 0.06</td>
<td>5.96 ± 0.18</td>
<td>1.29 ± 0.04</td>
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<td>Shock + al. epinephrine and amino guanidine</td>
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<td>4.23 ± 0.12</td>
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<td>4.28 ± 0.03</td>
<td>6.34 ± 0.12</td>
<td>1.48 ± 0.02*</td>
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<tr>
<td>Hemorrhagic shock + al. epinephrine and sulfasalazine</td>
<td>4</td>
<td>4.41 ± 0.08</td>
<td>6.52 ± 0.12</td>
<td>1.48 ± 0.01*</td>
</tr>
</tbody>
</table>

* Mean ± SEM; F/I Ratio: final over initial alveolar protein concentration ratio; *, p < 0.05 vs controls, #, p < 0.05 vs shock.
endothelial permeability to protein as indicated by the significant increase in extravascular plasma equivalents of noninstilled lung in hemorrhaged rats compared with controls (Table II).

Inhibition of iNOS in the lung restores normal alveolar epithelial fluid transport after hemorrhagic shock

NO has been shown to be one of the important oxidant radicals released during hemorrhagic shock and plays an essential role in the initiation of the inflammatory response in the lung (13). Thus, the second series of experiments was designed to determine whether the release of NO in the airspaces of the lung secondary to the expression of iNOS was responsible for the shock-mediated failure of the alveolar epithelium to respond to catecholamines. Hemorrhagic shock was also associated with a significant increase in the expression of iNOS protein in the lung homogenate of hemorrhaged rats compared with controls (Fig. 3). There was a significant increase in the production of nitrite, one of the major end-products of NO, by alveolar macrophages removed from hemorrhaged rats 6 h after onset of hemorrhagic shock and cultured ex vivo for 24 h in presence of increasing concentrations of LPS compared with controls (Fig. 4).

The next series of experiments was designed to determine whether the inhibition of iNOS either with amino guanidine or L-NIL, two specific inhibitors of iNOS, would restore the normal response of the alveolar epithelium to catecholamines. Rats pretreated either with amino guanidine or L-NIL underwent a hemorrhagic shock similar to that of nonpretreated rats. The quantity of blood removed, systemic arterial hypotension, metabolic acidosis, and calculated base deficit of the arterial blood at the end of the ischemic phase of hemorrhagic shock were comparable in both experimental groups.

Inhibition of iNOS in the lung with L-NIL significantly decreased the production of nitrite by alveolar macrophages removed from hemorrhaged rats 6 h after onset of hemorrhagic shock compared with the values measured in hemorrhaged, but not pretreated rats.

Table II. Effect of iNOS or NF-κB inhibition on the accumulation of extravascular plasma equivalents of the noninstilled lung measured 5 h after severe hemorrhagic shock*

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>n</th>
<th>Extravascular PE (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls − no shock</td>
<td>9</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Controls + alv. epinephrine</td>
<td>3</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Controls + alv. epinephrine and amino guanidine</td>
<td>4</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Controls + alv. epinephrine and L-NIL</td>
<td>4</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Controls + alv. epinephrine and PDTC</td>
<td>4</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Controls + alv. epinephrine and sulfasalazine</td>
<td>3</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Hemorrhagic shock</td>
<td>6</td>
<td>0.33 ± 0.10*</td>
</tr>
<tr>
<td>Hemorrhagic shock + alv. epinephrine</td>
<td>5</td>
<td>0.29 ± 0.08*</td>
</tr>
<tr>
<td>Hemorrhagic shock + alv. epinephrine and amino guanidine</td>
<td>4</td>
<td>0.08 ± 0.03*</td>
</tr>
<tr>
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<td>0.08 ± 0.02*</td>
</tr>
</tbody>
</table>

* Data as mean ± SEM, L-NIL: extravascular PE, extravascular plasma equivalents; *, p < 0.05 from controls; #, p < 0.05 vs shock. Lung endothelial permeability to protein was measured as accumulation of the vascular tracer, 131I-albumin in extravascular spaces of the noninstilled lung and was expressed as extravascular plasma equivalents.

FIGURE 2. Airspace instillation of de-AMP, but not direct activation of adenyl cyclase by forskolin, restores the ability of alveolar epithelium to respond to catecholamines by up-regulating alveolar fluid clearance after hemorrhage. Alveolar fluid clearance (% of instilled liquid) (mean ± SEM) is shown for control and hemorrhaged rats that did or did not have their airspaces instilled with de-AMP, a stable analog of cAMP (10−5 M) or forskolin, an agonist of adenyl cyclase (5 × 10−3 M). *, p < 0.05 from control rats that did not have their airspace instilled with epinephrine.

FIGURE 3. Representative Western blot analysis demonstrating increased expression of iNOS protein in the lung after hemorrhage. A, Western blot analysis for iNOS protein in control and hemorrhaged rats. One representative experiment is shown for each experimental condition. Four additional experiments gave comparable results. B. Densitometry analysis of five comparable Western blots for detection of iNOS protein showed a significant increase in the expression of iNOS protein in lung homogenate from hemorrhaged compared with control nonhemorrhaged rats. *, p < 0.05 from control rats.
were pretreated either with amino guanidine or L-NIL, specific iNOS inhibitors, or PDTC or sulfasalazine, specific inhibitors of the activation of NF-κB (Fig. 4). Pretreatment of control rats with either amino guanidine or L-NIL did not affect the ability of the alveolar epithelium to up-regulate vectorial fluid transport in response to catecholamines (Table I, Fig. 5). Comparable values were found when alveolar fluid clearance was measured with the 125I-labeled albumin (amino guanidine-pretreated rats: 43 ± 2%; L-NIL pretreated rats: 42 ± 3%). Finally, inhibition of iNOS in the lung was associated with a normalization of lung endothelial permeability to protein in hemorrhaged rats (Table II).

Pretreatment of control rats with either amino guanidine or L-NIL did not affect the ability of the alveolar epithelium to up-regulate vectorial fluid transport in response to catecholamines (Table I). Alveolar fluid clearance was significantly increased, as expected, in control rats pretreated with either amino guanidine (46 ± 1%) or L-NIL (45 ± 3%) and was comparable with that measured in control rats (44 ± 3%). Comparable values were found when alveolar fluid clearance was measured with the 125I-labeled albumin (data not shown). In addition, neither amino guanidine nor L-NIL affected the rate of alveolar fluid clearance in control rats that did not have their airspaces instilled with epinephrine (data not shown).

**Airspace instillation of dc-AMP, but not direct stimulation of adenyl cyclase with forskolin, restores normal alveolar epithelial fluid transport after hemorrhagic shock**

The third series of experiments was designed to determine whether NO would not only affect the function of the β-adrenergic receptor, but also that of other membrane proteins, such as adenyl cyclase, involved in the β-adrenergic receptor-cAMP signaling pathway. Forskolin, an adenyl cyclase agonist, was added to the protein solution instilled into the distal airspaces of hemorrhaged rats. Hemorrhaged rats instilled with dc-AMP, a stable analog of cAMP, served as positive controls. Rats pretreated either with forskolin or dc-AMP underwent a hemorrhagic shock similar to that of nonpretreated rats.

Direct stimulation of adenyl cyclase by forskolin in hemorrhaged rats did not restore the normal catecholamine-mediated up-regulation of alveolar liquid clearance. The final-to-initial distal airspace unlabeled protein concentration was 1.39 ± 0.04 in hemorrhaged rats instilled with forskolin vs 1.34 ± 0.02 in hemorrhaged rats not instilled with forskolin. Also, comparable values were obtained for alveolar fluid clearance measured either with the nonlabeled bovine albumin (Fig. 2) or with the 125I-labeled albumin (34 ± 2 vs 32 ± 2%, p < 0.05). In contrast, direct stimulation of adenyl cyclase by forskolin significantly increased vectorial fluid transport across the alveolar epithelium of control, nonhemorrhaged rats. The final-to-initial distal airspace unlabeled protein concentration was 1.55 ± 0.02, and alveolar fluid clearance measured either with the nonlabeled bovine albumin (Fig. 2) or with the 125I-labeled albumin (43 ± 2%) was significantly increased. Moreover, there was a significant increase in the final-to-initial distal airspace unlabeled protein concentration (1.52 ± 0.02 vs 1.39 ± 0.04) and the corresponding alveolar fluid clearance in hemorrhaged rats that had their airspaces instilled with dc-AMP compared with the values measured in hemorrhaged rats instilled with forskolin (Fig. 2).

**Inhibition of NF-κB in the lung restores normal alveolar epithelial fluid transport after hemorrhagic shock**

Activation of the proinflammatory transcriptional factor, NF-κB, occurs early (15 min) after the onset of septic (16) and hemorrhagic shock (17). Also, in vivo inhibition of NF-κB activation prevents endotoxin-dependent iNOS expression in the lung (18). Thus, the fourth series of experiments was designed to determine whether inhibition of activation of NF-κB in the lung by sulfasalazine or PDTC would affect the release of NO within the airspaces of the lung after prolonged hemorrhagic shock and would restore the physiological catecholamine-mediated up-regulation of alveolar liquid clearance. Rats pretreated either with sulfasalazine or PDTC underwent a hemorrhagic shock similar to that of nonpretreated rats.

Pretreatment with sulfasalazine significantly decreased the production of nitrite by alveolar macrophages removed from hemorrhaged rats 6 h after onset of hemorrhagic shock (Fig. 4). This decrease in the production of nitrite in hemorrhaged rats pretreated with inhibitors of the activation of NF-κB was associated with a restoration of the physiological catecholamine-mediated up-regulation of alveolar liquid clearance. The final-to-initial distal airspace unlabeled protein concentration and the corresponding alveolar fluid clearance were significantly increased in hemorrhaged rats pretreated either with sulfasalazine or PDTC compared with
the values measured in hemorrhaged, but not pretreated rats (Table I, Fig. 5). Comparable values were found when alveolar fluid clearance was measured with the 125I-labeled albumin (sulfasalazine-pretreated rats: 44 ± 2%; PDTC-pretreated rats: 43 ± 3%). Finally, pretreatment either with sulfasalazine or PDTC was associated with a normalization of lung endothelial permeability to protein in hemorrhaged rats (Table II).

Pretreatment of control rats with either sulfasalazine or PDTC did not affect the ability of the alveolar epithelium to up-regulate vectorial fluid transport in response to exogenous β-adrenergic agonists (Table I). Alveolar fluid clearance was also significantly increased in control rats pretreated either with sulfasalazine (45 ± 1%) or PDTC (43 ± 2%) and comparable with that measured in control, but not pretreated rats (44 ± 3%). Comparable values were found when alveolar fluid clearance was measured with the 125I-labeled albumin (data not shown). In addition, the results of preliminary studies indicate that neither sulfasalazine nor PDTC affected the rate of alveolar fluid clearance in control rats that did not have their airspaces instilled with epinephrine (data not shown).

To demonstrate that sulfasalazine inhibits NF-κB activation using an in vitro model relevant to these studies, we conducted several new experiments using A549 cells, a human alveolar epithelial cell line. In the first series of experiments, A549 cells were stimulated for 12 min with cytokine mix (a mixture of TNF-α, IFN-γ, and IL-1β), an in vitro model representative of the oxidative stress to the alveolar epithelium observed after hemorrhagic shock. Then, nuclear proteins were extracted and EMSA performed. Exposure to a mixture of proinflammatory cytokines for 12 min significantly increased nuclear translocation of NF-κB. Compared with the values measured in cells exposed to cytokine vehicle only, the intensity of the retarded κB oligonucleotide was increased ~6-fold in reactions containing nuclear extracts from cytokine-treated cells (Fig. 6). The specificity of the EMSA for NF-κB was determined by performing competitive reactions with unlabeled homologous κB. Inclusion of a 100-fold excess of the unlabeled homologous κB oligomer completely inhibited binding of the labeled κB oligonucleotide (Fig. 6). Exposure to proinflammatory cytokines also significantly increased NF-κB activation of a κ-dependent luciferase reporter construct (3xIgκBαLuc) (Fig. 7). Finally, cytokine-mediated increase in the nuclear translocation of NF-κB was associated with a complete degradation of cytoplasmic I-κBα protein in A549 cells (Fig. 8).

Pretreatment with sulfasalazine (2 μM) significantly decreased the nuclear translocation of NF-κB. Compared with the values measured in cells exposed to cytokines, the intensity of the retarded κB oligonucleotide was decreased by 30% in reactions containing nuclear extracts from sulfasalazine-pretreated cells (Fig. 6). Exposure to sulfasalazine also completely abolished NF-κB activation of a κ-dependent luciferase reporter construct (Fig. 7). Finally, pretreatment with sulfasalazine significantly attenuated the cytokine-mediated degradation of cytoplasmic I-κBα in A549 cells (Fig. 8). In summary, these experiments indicate that sulfasalazine decreases cytokine-mediated translocation of NF-κB to the nucleus and activation of NF-κB-responsive genes.

**Discussion**

The primary objective of this study was to investigate the mechanisms responsible for the oxidant-mediated inhibition of alveolar epithelial fluid transport after severe hemorrhagic shock, one of the common causes of acute lung injury and pulmonary edema in humans (1). The major findings can be summarized as follows. First, release of NO secondary to an increase in the expression of iNOS in the lung diminishes the capacity of the alveolar epithelium to actively transport fluid from the airspaces after severe hemorrhage. Second, NO preferentially inhibits alveolar epithelial fluid transport regulated by cAMP-dependent mechanisms by directly affecting the function of the β-adrenergic receptor and adenyl cyclase. Third, shock-mediated release of NO in the airspaces of the lung depends in part on the activation and nuclear translocation of NF-κB.

Preservation of the capacity of the alveolar epithelium to actively remove fluid from the airspaces is critical for the survival of patients with acute lung injury (21). Therefore, we developed an experimental animal model to investigate the mechanisms that control alveolar epithelial fluid transport under clinically relevant pathological conditions responsible for the development of acute lung injury in humans, such as hemorrhage or sepsis. The release of
endogenous catecholamines up-regulated alveolar epithelial fluid transport by cAMP-dependent mechanisms and prevented alveolar flooding after onset of short-term septic or hemorrhagic shock (6–8). However, this normal up-regulation of alveolar liquid clearance was inhibited by a neutrophil-dependent oxidative injury to the alveolar epithelium after severe hemorrhage (9), although the molecular mechanisms responsible for the oxidative injury to the alveolar epithelium were unknown.

Several lines of evidence indicate that induced NO may contribute to the inflammatory response and subsequent end-organ damage in the lung after severe hemorrhage. First, hemorrhagic shock produced a time-dependent increase in iNOS activity in the lung (14, 15). Second, shock-mediated expression of some proinflammatory mediators, such as IL-6 and G-CSF, are iNOS dependent because their expression was inhibited by pretreatment with L-NIL and was absent in iNOS knockout mice (13). Third, inhibition of iNOS by pretreatment with L-NIL in hemorrhaged rats was associated with a significant decrease in the shock-dependent neutrophil infiltration in the lung and with a significant reduction in the shock-mediated increase in extravascular lung water (13). In addition, selective inhibition of iNOS attenuated lung damage in acute lung injury caused by endotoxin (22–24). Thus, the first objective of the study was to determine whether the release of NO in the airspaces of the lung was responsible for the decrease in the alveolar epithelial fluid transport after severe hemorrhage. Inhibition of iNOS by two unrelated iNOS inhibitors prevented the hemorrhage-dependent increase in release of nitrite by alveolar macrophages and restored the normal fluid transport capacity across the alveolar epithelium after severe hemorrhagic shock. In addition, inhibition of iNOS caused a normalization of shock-mediated increase in lung endothelial permeability to protein. Thus, these results provide the first in vivo evidence that induction of NO synthesis in the lung is a critical mechanism responsible for the inhibition of the cAMP-dependent up-regulation of alveolar epithelial fluid transport after severe hemorrhage. It is unlikely that the protective effect observed in hemorrhaged and pretreated rats was due to a nonspecific effect of iNOS inhibitors. Amino guanidine can have effects that are unrelated to inhibition of iNOS activity, such as inhibition of diamine oxidase and nonenzymatic glycosylation. However, both iNOS inhibitors are chemically unrelated and both restored a normal alveolar epithelial fluid transport after severe hemorrhage. Moreover, neither amino guanidine nor L-NIL affected alveolar fluid clearance in control rats.

To further explore how NO decreases the capacity of the alveolar epithelium to remove fluid from the airspaces after prolonged hemorrhagic shock, the second objective was to determine whether shock-dependent release of induced NO would also affect the function of other membrane proteins involved in the β-adrenergic receptor-cAMP signaling pathway in the alveolar epithelium. Airspace instillation of dc-AMP, but not direct stimulation of adenyl cyclase by forskolin, up-regulated vectorial fluid transport across the alveolar epithelium following hemorrhage. In contrast, both dc-AMP and forskolin stimulated alveolar fluid transport in control nonhemorrhaged rats. Thus, these results provided good evidence that the shock-mediated release of induced NO can alter the function of membrane proteins involved in the β-adrenergic receptor-cAMP signaling pathway in the alveolar epithelium.

There are several mechanisms that could explain why inhibition of induced NO restores cAMP-dependent alveolar epithelial fluid transport after prolonged hemorrhagic shock. The first mechanism could involve NO-mediated inhibition of cation channels on rat alveolar type II cells by cGMP-dependent and -independent pathways. For example, 5-nitrosoglutathione, a NO donor, suppressed the activity of nonselective cation channels on the apical surface of rat alveolar type II cells, an effect mediated by a cGMP-dependent protein kinase (25). Also, NO released by NO donors directly inhibited amiloride-sensitive sodium channels and NaKATPase in confluent rat alveolar type II cell monolayers by a cGMP-independent mechanism (26). Recently, peroxynitrite, a strong oxidant...
produced by the reaction between NO and superoxide, has been reported to decrease amiloride-sensitive sodium current in oocytes expressing αβγ-ENaC by a mechanism related to the oxidation of critical amino acid residues in the ENaC protein (27). The second mechanism could involve an effect of NO on chloride secretion in lung epithelial cells. NO has been shown to activate noncystic fibrosis transmembrane conductance regulator (CFTR) chloride channels in human lung epithelial cells (A549) by a GMP-dependent mechanism (28). In addition, the results of a recent study indicate that catecholamines and agents that increase cAMP can cause a transient increase in chloride secretion by the alveolar epithelium of rabbits in vivo (29). Thus, the release of induced NO within the airspaces of hemorrhaged rats could result in an upregulated cytokine expression and an increased cytokine expression in the lung (17). At later time points, cyclic AMP response element binding protein (CREB)/NF-κB interactions and increased cytokine expression may contribute to the xanthine oxidase-dependent activation of NF-κB (31). Second, there is experimental evidence that NF-κB activation is required for cytokine induction of iNOS protein. Indeed, a recent study has identified four NF-κB enhancer elements upstream in the human iNOS promoter that confer inducibility to TNF-α and IL-1β (32). Also, earlier studies have shown that the iNOS gene is transcriptionally regulated by NF-κB-dependent mechanisms (33, 34), although in some cells, such as alveolar macrophages, there is also a cAMP-mediated NF-κB-dependent pathway for iNOS activation (35). Moreover, the results of a recent study indicated that low pH, as observed in hemorrhagic shock, may increase the expression of iNOS through the activation of NF-κB in macrophages (36). Third, the results presented here are the first in vivo evidence that the activation of NF-κB may contribute to the shock-mediated decrease in the ability of the alveolar epithelium to actively remove fluid from the airspaces after prolonged hemorrhagic shock.

For several reasons, it is unlikely that the protective effect observed in hemorrhaged and pretreated rats was due to a nonspecific effect of PDTC or sulfasalazine. First, although PDTC has antioxidant effects unrelated to inhibition of the activation of NF-κB that could contribute to the observed effect in our experimental model (37), both chemically unrelated NF-κB inhibitors, PDTC and sulfasalazine, restored a normal alveolar epithelial fluid transport after severe hemorrhage. In addition, in contrast to its salicylate moiety, 5-aminosalicylic acid, sulfasalazine has recently been shown in vitro to be a specific inhibitor of the activation of NF-κB by interfering with IκBα phosphorylation (19, 30). Second, neither PDTC nor sulfasalazine affected alveolar fluid clearance in control rats.

There are several lines of evidence that the activation of NF-κB in the airspaces of the lung may be an important factor in the development of the inflammatory response in the lung after fluid resuscitation from prolonged hemorrhagic shock. First, previous studies have shown that hemorrhage leads to a rapid in vivo activation in the lung of NF-κB through xanthine oxidase-dependent and independent mechanisms (31). Hemorrhage is followed by two phases of NF-κB activation. The first phase, which is xanthine oxidase-dependent, occurs immediately after blood loss. Hemorrhage produces intense pulmonary vasoconstriction, local release of catecholamines, and increased generation of reactive oxygen intermediates that can activate NF-κB (17). At later time points, cyclic AMP response element binding protein (CREB)/NF-κB interactions and increased cytokine expression may contribute to the xanthine oxidase-dependent activation of NF-κB (31). However, a direct effect of NO on the apical cation channels of the alveolar epithelium might be more critical amino acid residues in the ENaC protein (27). The second mechanism could involve an effect of NO on chloride secretion by the alveolar epithelium of rabbits in vivo (29). Thus, the release of induced NO within the airspaces of hemorrhaged rats could result in an upregulated cytokine expression and an increased cytokine expression in the lung (17). At later time points, cyclic AMP response element binding protein (CREB)/NF-κB interactions and increased cytokine expression may contribute to the xanthine oxidase-dependent activation of NF-κB (31). Second, there is experimental evidence that NF-κB activation is required for cytokine induction of iNOS protein. Indeed, a recent study has identified four NF-κB enhancer elements upstream in the human iNOS promoter that confer inducibility to TNF-α and IL-1β (32). Also, earlier studies have shown that the iNOS gene is transcriptionally regulated by NF-κB-dependent mechanisms (33, 34), although in some cells, such as alveolar macrophages, there is also a cAMP-mediated NF-κB-dependent pathway for iNOS activation (35). Moreover, the results of a recent study indicated that low pH, as observed in hemorrhagic shock, may increase the expression of iNOS through the activation of NF-κB in macrophages (36). Third, the results presented here are the first in vivo evidence that the activation of NF-κB may contribute to the shock-mediated decrease in the ability of the alveolar epithelium to actively remove fluid from the airspaces after prolonged hemorrhagic shock.

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Clinical implications

What is the clinical importance of the rate of alveolar epithelial fluid transport in patients with acute lung injury? The results of earlier clinical studies indicated that the presence of intact alveolar liquid clearance early after the onset of hydrostatic or high permeability edema was associated with a shorter duration of mechanical ventilation and a better overall outcome (21). However, the rate of alveolar liquid clearance has recently been measured in a large number of patients with acute lung injury and hydrostatic pulmonary edema. Results of these clinical studies indicate that 50–60% of the patients with pulmonary edema have impaired or submaximal alveolar liquid clearance (38, 39). Moreover, impaired or submaximal alveolar liquid clearance was an early predictor of prolonged duration of mechanical ventilation and higher hospital mortality in patients with acute lung injury (38). The mechanisms responsible for this reduction in the rate of alveolar liquid clearance have not been adequately explored, although the results of a recent clinical study revealed up-regulated production of reactive-oxygen-nitrogen intermediates in the alveolar spaces of patients with acute lung injury that was associated with impaired alveolar fluid clearance (40). Thus, this study provides new insights regarding the potential contribution of a NO-dependent oxidant pathway that can inhibit alveolar epithelial fluid transport in vivo following severe hemorrhagic shock, an important cause of acute lung injury in humans.

In summary, these results provide the first in vivo evidence that NO, released within the airspaces secondary to the NF-κB-dependent activation of iNOS, is a major proximal mediator that can limit the rate of alveolar epithelial transport after prolonged hemorrhagic shock by altering the function of membrane proteins involved in the β-adrenergic receptor-CAMP signaling pathway in the alveolar epithelium. This mechanism may be important to explain the clinical evidence that some patients with acute lung injury have impaired rates of alveolar epithelial fluid clearance.

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