TNF-α stimulates alveolar liquid clearance during intestinal ischemia-reperfusion in rats

ANNA BÖRJESSON, ANDREAS NORLIN, XIANGDONG WANG, ROLAND ANDERSSON, AND HANS G. FOLKESSON. TNF-α stimulates alveolar liquid clearance during intestinal ischemia-reperfusion in rats. Am. J. Physiol. Lung Cell. Mol. Physiol. 278: L3–L12, 2000.—Intestinal ischemia-reperfusion commonly occurs in critically ill patients and may lead to the development of remote organ injury, frequently involving the lungs. In the present study, alveolar liquid clearance was studied in ventilated, anesthetized rats subjected to 45 min of intestinal ischemia followed by 3 h of reperfusion. An isosmolar 5% albumin solution was instilled into the lungs, and alveolar liquid clearance was measured from the increase in alveolar protein concentration as water was reabsorbed over 45 min. Intestinal ischemia-reperfusion resulted in a 76% increase in alveolar liquid clearance compared with the control value (P < 0.05). The stimulated alveolar liquid clearance seen after intestinal ischemia-reperfusion was not inhibited by propranolol, indicating stimulation through a noncatecholamine-dependent pathway. Intestinal ischemia-reperfusion did not result in increased intracellular cAMP levels. Amiloride inhibited similar fractions in animals subjected to ischemia-reperfusion and control animals. Administration of a neutralizing polyclonal anti-tumor necrosis factor-α antibody before induction of intestinal ischemia completely inhibited the increased alveolar liquid clearance observed after intestinal ischemia-reperfusion. In conclusion, our results suggest that intestinal ischemia-reperfusion in rats leads to stimulation of alveolar liquid clearance and that this stimulation is mediated, at least in part, by a tumor necrosis factor-α-dependent mechanism.

tumor necrosis factor-α; endothelium; epithelium; lung; lung injury; pulmonary edema

Intestinal ischemia-reperfusion is a potentially life-threatening condition commonly associated with injury to organs remote from the initial insult (9, 15, 17). The remote organ injury is suggested to be caused by release of inflammatory mediators including arachidonic acid metabolites, reactive oxygen species, complement factors, and cytokines such as tumor necrosis factor (TNF)-α (5, 36, 40). It has previously been demonstrated that TNF-α is an essential cytokine in the cascade that causes lung endothelial injury after lung ischemia-reperfusion (13). Also, leukocytes have been suggested as important mediators of both local intestinal injury (10) and remote tissue injury (29, 31). Clinical studies as well as animal models have demonstrated an impairment of pulmonary function after intestinal ischemia-reperfusion (15, 19, 29). Injury to the intestinal barrier during ischemia-reperfusion may lead to translocation of bacteria and bacterial products such as endotoxin, with the systemic dissemination linked to the development of remote tissue injury (15, 35). Endotoxin can stimulate macrophages to synthesize and secrete several cytokines and other inflammatory mediators including TNF-α (19).

A previous study (38) has implicated TNF-α as a mediator of hemodynamic dysfunction secondary to intestinal ischemia-reperfusion in rats. TNF-α has also been demonstrated to increase pulmonary endothelial permeability secondary to acute bacterial lung inflammation in rats (18). Furthermore, TNF-α has been reported to increase sodium-coupled amino acid transport in rat hepatocytes (25), thus suggesting that TNF-α may modulate vectorial solute transport across other cell membranes. A recent study by Rezaiguia et al. (28) demonstrated that TNF-α stimulates alveolar liquid clearance in rats during bacterial pneumonia from Pseudomonas aeruginosa. Furthermore, several studies have demonstrated that β-adrenergic stimulation increases alveolar liquid clearance during pathological conditions (27) as well as under normal conditions (7, 23).

We hypothesized that lung injury after intestinal ischemia-reperfusion is caused by an increase in pulmonary endothelial-epithelial permeability. Furthermore, we hypothesized that TNF-α protects the lungs from a rapid lethal flooding by stimulating alveolar liquid clearance. Therefore, our first objective was to investigate alveolar barrier permeability and alveolar liquid clearance after intestinal ischemia-reperfusion injury. Because intestinal ischemia-reperfusion stimulated alveolar liquid clearance and because several authors (27) have shown that endogenous epinephrine can stimulate alveolar liquid clearance in a variety of pathological conditions, our second objective was to investigate the role of endogenous catecholamines and their intracellular second messenger, cAMP, on alveolar liquid clearance and alveolar epithelial permeability after intestinal ischemia-reperfusion. Because β-adrenergic stimulation was not responsible for the stimulated alveolar liquid clearance after intestinal...

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ischemia-reperfusion and because TNF-α has been demonstrated to increase alveolar liquid clearance under other pathological conditions (28), our third objective was to study whether TNF-α may stimulate alveolar liquid clearance after intestinal ischemia-reperfusion.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (n = 49; B&K Universal, Sollentuna, Sweden) weighing 230–550 g were fed standard rat chow (R 3, Astra-Ewos, Södertälje, Sweden) and had tap water ad libitum. The rats were maintained in a 12:12-h day-night rhythm at a temperature of 20 ± 2°C and a relative humidity of 50 ± 10%. The Ethical Review Committee on Animal Experiments at Lund University (Lund, Sweden) approved the experimental protocol.

Preparation of Instillates and Rhodamine B Isothiocyanate-Conjugated Dextran Injection Solution

A 5% albumin solution was prepared by dissolving 50 mg/ml of bovine serum albumin (Sigma, St. Louis, MO) in 0.9% NaCl. A sample of the instillate was saved for total protein measurement. In some studies (see Specific Experimental Protocols), the β-adrenergic antagonist propranolol (10⁻⁶ M; Sigma) or the sodium-channel inhibitor amiloride (10⁻³ M; Sigma) was added to the instillate solution.

Rhodamine B isothiocyanate (RITC)-conjugated Dextran 70 (RITC-dextran; mol wt 70,000; Sigma) was dissolved at a concentration of 35 mg/ml in 2.5 ml of 0.9% NaCl and filtered through a PD-10 column (Pharmacia Bioscience, Uppsala, Sweden) to separate free unbound RITC molecules from the RITC-dextran. The filtered RITC-dextran was then diluted with isosmolar 0.9% NaCl to a final concentration of 2.5 mg/ml.

Induction of Intestinal Ischemia-Reperfusion

The rats were anesthetized with an intramuscular injection of ketamine (100 mg/kg body weight; Ketalar, Parke-Davis, Barcelona, Spain) and xylazine (10 mg/kg body weight; Rompun, Bayer, Leverkusen, Germany). Through a midline laparotomy, the superior mesenteric artery was located and isolated from the surrounding tissues, and a vascular clip was placed around the vessel near the aortic origin. The abdominal incision was then temporarily closed, and the rat was placed on a heating pad during the ischemic period. After 45 min, the abdominal incision was opened, and the vascular clip was removed. Reperfusion was noted by visual inspection of the intestine in each animal, after which the abdominal incision was permanently closed. Reperfusion was continued for 3 h. A sham operation on control rats was carried out as above but without placing the vascular clip on the superior mesenteric artery.

Surgical Procedures and Ventilation

One hour fifteen minutes after the onset of reperfusion, the animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight; Apoteksbolaget, Umeå, Sweden). A 2.0-mm-ID endotracheal tube (PE-240; Clay Adams, Becton Dickinson, Sparks, MD) was inserted into the trachea through a tracheostomy. A 0.58-mm-ID catheter (PE-50; Clay Adams, Becton Dickinson) was inserted into the right carotid artery to monitor systemic blood pressure, administer drugs, and obtain blood samples. Pancuronium bromide (0.3 mg·kg body wt⁻¹·h⁻¹; Pavulon, Organon Teknika, Boxtel, The Netherlands) was administered through the arterial catheter for neuromuscular blockade, and then the animals were immediately connected to and ventilated with a constant-volume piston pump (Harvard Apparatus, Nantucket, MA). Ventilation was carried out with an inspired oxygen fraction of 1.0 and a tidal volume just sufficient to reach a peak airway pressure of 10–12 cmH₂O and a positive end-expiratory pressure of 3 cmH₂O during the baseline period.

Peak airway pressure, blood pressure, and heart rate were measured with calibrated pressure transducers (UFI model 1050BP; BioPac Systems, Goleta, CA) connected to analog-to-digital converters and amplifiers (MP100 and DA100, respectively; BioPac Systems) and continuously recorded on an IBM-compatible computer with Acknowledge 3.2.4 software (BioPac Systems).

General Experimental Protocol

After the surgical procedures, the animals were placed in the left decubitus position on a slanting board. A heating pad covered the animals during the experiment to control and maintain normal body temperature. A 30-min baseline period with stable blood pressure and heart rate was required before fluid instillation into the lungs. Ten minutes into the baseline period, RITC-dextran was injected intra-arterially (2 ml/kg body weight, resulting in ~ 0.07 mg RITC-dextran/ml blood). A blood sample (1 ml) was taken 10 min after the RITC-dextran injection.

After the 30-min baseline period, an instillation catheter (PE-50; Clay Adams, Becton Dickinson) was gently passed through the endotracheal tube and advanced to a wedged position in the distal air spaces of the lungs. Then, 0.8 ml of the 5% albumin solution was instilled over 20 min into the lungs. The instillation catheter was withdrawn after the instillation was completed.

Forty-three minutes after instillation, a 5-ml blood sample was taken, and at 45 min, the animal was given an overdose (30 mg) of pentobarbital sodium intra-arterially. The abdomen was opened, and the rats were exsanguinated by transecting the abdominal aorta. The lungs and heart were carefully removed en bloc from the thorax through a midline sternotomy. A PE-50 catheter (Clay Adams, Becton Dickinson) was gently passed to a wedged position in the instilled lung, and a sample of the remaining alveolar liquid was aspirated. The lungs were then homogenized for fluorescence measurements and wet-to-dry weight determinations. The blood samples were centrifuged at 4,000 g for 5 min, and the plasma was collected for analysis of fluorescence, total protein concentration, and TNF-α measurements.

Specific Experimental Protocols

Group 1: Control rats. Rats (n = 4) were sham operated as described in Surgical Procedures and Ventilation. After the baseline period, the rats were instilled with the 5% albumin solution, studied for 45 min, and processed as described in General Experimental Protocol. A separate set of control rats (n = 2) was injected with a nonspecific IgG antibody to control for possible nonspecific effects from the anti-TNF-α monoclonal antibody (MAB) injection (see Group 4: Amiloride studies). The animals were treated and instilled as described in General Experimental Protocol. Because there were no effects on any of the measured parameters from the nonspecific IgG, these rats were combined with the sham-operated control rats, giving a total of six rats. These rats are referred to as the control rats. Three separate rats were subjected to a sham
operation, and their lungs were perfused free of blood. cAMP production was determined in tissue samples over 10 min in 37°C as described in Pulmonary cAMP Generation.

Group 2: Intestinal ischemia-reperfusion. Rats (n = 5) were subjected to 45 min of intestinal ischemia followed by 3 h of reperfusion as described in Surgical Procedures and Ventilation. After the baseline period, the rats were instilled with the 5% albumin solution and studied for 45 min. The rats were then treated as described in General Experimental Protocol. A separate set of rats (n = 4) was injected with a nonspecific IgG antibody to study possible unspecific antibody effects from the anti-TNF-α MAb injection (see Group 4: Amiloride studies). The animals were treated and instilled as described in General Experimental Protocol. Because no effects from the nonspecific IgG were observed on any of the studied parameters, the rats were combined with the regular ischemia-reperfusion rats, giving a total of nine rats, and are referred to as the ischemia-reperfusion group. Three additional rats were subjected to 45 min of intestinal ischemia followed by 3 h of reperfusion, and their lungs were perfused free of blood. cAMP production was determined in tissue samples over 10 min in 37°C as described in Pulmonary cAMP Generation.

Group 3: Propranolol studies. Rats (n = 4) were subjected to 45 min of intestinal ischemia followed by 3 h of reperfusion as described in Surgical Procedures and Ventilation. After the baseline period, the rats were instilled with the 5% albumin solution containing 10⁻⁴ M propranolol. The rats were studied for 45 min and processed as described in General Experimental Protocol. A separate set of rats (n = 5) was sham operated as described in Surgical Procedures and Ventilation and instilled with the 5% albumin solution containing 10⁻⁴ M propranolol. The rats were studied for 45 min and processed as described in General Experimental Protocol.

Group 4: Amiloride studies. Rats (n = 4) were subjected to 45 min of intestinal ischemia followed by 3 h of reperfusion as described in Surgical Procedures and Ventilation. After the baseline period, the rats were instilled with the 5% albumin solution containing 10⁻³ M amiloride. Amiloride was used at 10⁻³ M because ~50% is bound to the protein in the instillate, and because of its relatively low molecular weight, amiloride leaves the air spaces rapidly (24, 39). The rats were studied for 45 min and processed as described in General Experimental Protocol. A separate set of rats (n = 4) underwent a sham operation as described in Surgical Procedures and Ventilation and instilled with the 5% albumin solution containing 10⁻³ M amiloride. The rats were studied for 45 min and processed as described in General Experimental Protocol.

Group 5: TNF-α inhibition studies. A neutralizing anti-TNF-α MAb (0.1 ml; IP-400, Genzyme, Cambridge, MA) originally directed against mouse TNF-α, which also neutralized rat TNF-α, was administered intracardially to the rats (n = 6) 30 min before induction of ischemia-reperfusion. After the baseline period, the rats were instilled with the 5% albumin solution and studied for 45 min. The rats were then processed as described in General Experimental Protocol.

Alveolar Liquid Clearance

The increase in alveolar concentration of the instilled protein over 45 min was used to measure the clearance of liquid from the distal air spaces (across the alveolar epithelium and distal airway epithelium) as done before (4, 7, 8, 11, 21, 23). Data on alveolar liquid clearance are shown in two ways. First, alveolar liquid clearance is presented as a ratio of final aspirated alveolar fluid protein concentration to instilled fluid protein concentration. The final-to-instilled protein concentration ratio provides direct evidence for alveolar liquid clearance because liquid must be transported from the air spaces for the final alveolar protein concentration to rise. Because there were small changes in epithelial and endothelial permeabilities to protein (i.e., very little protein left the air spaces in any of the groups; see RESULTS), this method is accurate for measuring liquid clearance from the distal air spaces of the lungs. The second method is based on calculating alveolar liquid clearance (ALC; expressed as a percentage of instilled volume) with Eq. 1

\[
ALC = \left[ \frac{(V_f - V_i)/V_i} \right] \times 100
\]

where \(V_i\) is the instilled volume and \(V_f\) is the final alveolar volume (calculated from the protein concentrations in the instilled and final alveolar liquids, respectively). The term alveolar does not, however, imply that all reabsorption of liquid occurs at the alveolar level; i.e., some liquid reabsorption may occur across the distal bronchial epithelium because it can also transport sodium (3).

Endothelial Permeability to Protein

To estimate endothelial permeability to protein, the clearance of the vascular tracer RITC-dextran into the extravascular compartments of the lungs (interstitium and air spaces) was measured. The total extravascular RITC-dextran accumulation in the alveolar liquid and the lung homogenate was measured spectrophotofluorometrically (CytoFluor 2300, Millipore, Bedford, MA) and is expressed as extravascular plasma equivalents. Passage of RITC-dextran across the endothelial epithelial barrier was considered to be equal to that of albumin because they have similar molecular weights (70,000 vs. 67,000). The calculation of endothelial protein passage was done with the RITC-dextran concentration in the different compartments and applying them in Eq. 2

\[
\text{RITC-dextran}_{\text{extravascular}} = \frac{\text{RITC-dextran}_{\text{total}}}{\text{RITC-dextran}_{\text{vascular space}}}
\]

where RITC-dextran_{extravascular} is the RITC-dextran concentration in the extravascular compartments of the lung, RITC-dextran_{total} is the total RITC-dextran concentration in the lung, and RITC-dextran_{vascular space} is the RITC-dextran concentration in the vascular compartment in the lung. To calculate RITC-dextran_{vascular space}, the RITC-dextran measured in the last plasma sample was multiplied by the blood volume in the lungs corrected for hematocrit. The blood volume (Q_Bi) in the lungs at the end of the experiment was calculated from Eq. 3

\[
Q_{Bi} = 1.039 \times ([Q_H \times FW_H \times Hb_S]/[FW_S \times Hb_Bi])
\]

where 1.039 is the density of blood in grams per milliliter, \(Q_H\) is the weight of lung homogenate, \(FW_H\) is the fraction of water in the lung homogenate, Hb_S and FW_S are the hemoglobin concentration and fraction of water, respectively, in the supernatant obtained after centrifugation of the lung homogenate, and Hb_Bi is the hemoglobin concentration in the last blood sample. The fraction of water in the lung was obtained by gravimetric measurements of the lung as done before (4, 11, 21).

A ratio between the RITC-dextran concentration in the final alveolar fluid sample and the RITC-dextran plasma concentration provided an index of equilibration of the vascular protein tracer into the alveolar compartment as in an earlier experimental study of epithelial permeability (37).
After 2 h 30 min of reperfusion, the rats were anesthetized free of blood with 30 ml of 0.9% NaCl containing 10 M aminophylline (Sigma) at 15 cmH2O pressure via the pulmonary artery. The lungs were then removed and placed on ice until further processing. Duplicate samples of blood-free distal lung tissue weighing 28.0 ± 2.3 (SD) mg were incubated in 0.25 ml of 10 M aminophylline buffer as done before (23). Basal cAMP content was determined after 10 min of incubation at 4°C, and basal cAMP production was measured after 10 min of incubation at 37°C. Stimulated cAMP production was studied after 10 min at 37°C with 10 M forskolin (stimulates adenylate cyclase without receptor involvement) in 2.5% DMSO (Sigma). DMSO at this concentration has previously been demonstrated to have no effect on cAMP production (23). All reactions were stopped by adding 0.25 ml of trichloroacetic acid (Sigma) to the incubation buffer. The samples were homogenized and centrifuged at 4,000 g for 15 min at 4°C, and the supernatants were extracted with ether (5:1) three consecutive times to remove the trichloroacetic acid. The remaining ether was evaporated in a water bath at 70°C for 30 min. The samples were stored at −70°C until analysis. The concentration of cAMP in the samples was determined with a commercially available radioimmunoassay kit (NEN-DuPont, Boston, MA).

Pulmonary cAMP Generation

Lungs from six rats were used for the determination of pulmonary cAMP levels after sham operation (n = 3) and after intestinal ischemia followed by reperfusion (n = 3). After 2 h 30 min of reperfusion, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight; Apoteksbolaget) and 2,000 IU of heparin (Lövens, Ballerup, Denmark). An endotracheal tube (2.0 mm ID, PE-240) was inserted into the trachea through a tracheostomy. The lungs and heart were exposed through a midline sternotomy. The lungs were mechanically ventilated with a constant-volume piston pump (Harvard Apparatus, Natick, MA). After removal of the left atrium, the lungs were perfused from the carotid artery via the pulmonary artery. The lungs were then removed and placed on ice until further processing. Duplicate samples of blood-free distal lung tissue weighing 28.0 ± 2.3 (SD) mg were incubated in 0.25 ml of 10 M aminophylline buffer as done before. Basal cAMP content was determined after 10 min of incubation at 4°C, and basal cAMP production was measured after 10 min of incubation at 37°C. Stimulated cAMP production was studied after 10 min at 37°C with 10 M forskolin (stimulates adenylate cyclase without receptor involvement) in 2.5% DMSO (Sigma). DMSO at this concentration has previously been demonstrated to have no effect on cAMP production (23). All reactions were stopped by adding 0.25 ml of trichloroacetic acid (Sigma) to the incubation buffer. The samples were homogenized and centrifuged at 4,000 g for 15 min at 4°C, and the supernatants were extracted with ether (5:1) three consecutive times to remove the trichloroacetic acid. The remaining ether was evaporated in a water bath at 70°C for 30 min. The samples were stored at −70°C until analysis. The concentration of cAMP in the samples was determined with a commercially available radioimmunoassay kit (NEN-DuPont, Boston, MA).

TNF-α Analysis

Plasma from the last blood sample from rats in the control group, the intestinal ischemia-reperfusion group, and the intestinal ischemia-reperfusion plus anti-TNF-α MAb group was analyzed for TNF-α concentrations. An additional four animals were used for determining the time course of TNF-α release during intestinal ischemia-reperfusion. These rats were anesthetized by intraperitoneal pentobarbital sodium, and the right carotid artery was catheterized. Alveolar fluid instillation was not done in these rats. One milliliter of blood was taken from the carotid artery at the following time points: immediately before induction of ischemia (baseline), after 40 min of ischemia (ischemia alone), and 5, 15, 30, 60, and 90 min after the start of reperfusion. The collected blood volume was replaced by an equal volume of sterile 0.9% NaCl. The blood was centrifuged at 4,000 g at +4°C for 5 min and stored at −70°C until analysis.

Plasma levels of TNF-α were determined with a commercially available enzyme-linked immunosorbent assay specific for rat TNF-α (Genzyme). Intra- and interassay coefficients of variation were 5.15 and 5.15%, respectively.

Statistics

All data are means ± SD. The data were analyzed with Student's t-test or one-way analysis of variance, with Tukey's test post hoc when appropriate. Differences were considered significant when P < 0.05 was reached.

RESULTS

Alveolar Liquid Clearance Under Basal Conditions and After Intestinal Ischemia-Reperfusion

Anesthetized ventilated rats were instilled with 5% bovine serum albumin in 0.9% NaCl. After 45 min, a sample of the instilled solution was aspirated from the distal air spaces. The increase in total protein concentration during the 45-min period was used as a measurement of the liquid that had been cleared from the distal air spaces of the lungs. Intestinal ischemia followed by reperfusion resulted in a significant increase in alveolar liquid clearance by 76% compared with that in sham-operated control rats (P < 0.05; Fig. 1, Table 1).

Effect of Propranolol on Alveolar Liquid Clearance

To investigate whether the increased alveolar liquid clearance after intestinal ischemia-reperfusion was mediated by β-adrenergic-receptor activation from endogenous release of catecholamines, anesthetized ventilated rats were subjected to sham operation or intestinal I/R. The blood was centrifuged at 4,000 g for 5 min and stored at −70°C until analysis. The concentration of cAMP in the samples was determined with a commercially available radioimmunoassay kit (NEN-DuPont, Boston, MA).

Table 1. Alveolar liquid clearance over 45 min in rats subjected to sham operation or intestinal I/R

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Instilled</th>
<th>Final</th>
<th>Alveolar Liquid Clearance, %instilled volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>5.56 ± 0.80</td>
<td>6.28 ± 0.80</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>I/R</td>
<td>9</td>
<td>5.49 ± 0.82</td>
<td>6.93 ± 1.14</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>Control + propranolol (10⁻⁴ M)</td>
<td>6</td>
<td>5.58 ± 0.78</td>
<td>6.39 ± 0.86</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>I/R + propranolol (10⁻³ M)</td>
<td>4</td>
<td>5.31 ± 0.54</td>
<td>6.60 ± 0.48</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Control + amiloride (10⁻³ M)</td>
<td>4</td>
<td>5.42 ± 0.32</td>
<td>5.87 ± 0.07</td>
<td>8 ± 1*</td>
</tr>
<tr>
<td>I/R + amiloride (10⁻³ M)</td>
<td>4</td>
<td>5.65 ± 0.05</td>
<td>6.44 ± 0.58</td>
<td>12 ± 8</td>
</tr>
<tr>
<td>I/R + TNF-α MAb</td>
<td>6</td>
<td>6.61 ± 0.31</td>
<td>7.63 ± 0.55</td>
<td>13 ± 6†</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of rats. I/R, 45 min of intestinal ischemia followed by 3 h of reperfusion; TNF-α, tumor necrosis factor-α; MAb, monoclonal antibody; control, sham operated. Significantly different (P < 0.05) from: *control and control + propranolol; †I/R.
lated rats (control animals and animals subjected to intestinal ischemia-reperfusion) were instilled with the 5% albumin solution containing the β-antagonist propranolol (10⁻⁴ M). The addition of propranolol did not affect alveolar liquid clearance either in control animals or after intestinal ischemia-reperfusion compared with that in rats instilled with 5% albumin without propranolol (Fig. 2, Table 1).

Effect of Intestinal Ischemia-Reperfusion on Pulmonary cAMP Generation

Lung tissue samples from three sham-operated rats and three rats subjected to intestinal ischemia-reperfusion were incubated at 37°C for 10 min to determine the generation of intracellular cAMP under normal (unstimulated) conditions and after stimulation with 10⁻⁴ M forskolin. All cAMP production values were corrected for basal cAMP content measured over 10 min at 4°C.

There was no increase in intracellular cAMP levels after intestinal ischemia-reperfusion compared with that in sham-operated control animals (7.74 ± 1.33 vs. 7.77 ± 1.87 pmol/l). Stimulation of adenylate cyclase with forskolin (10⁻⁴ M) raised the intracellular cAMP levels over 10 min compared with the basal levels in both the sham-operated and intestinal ischemia-reperfusion animals as expected because forskolin acts directly on adenylate cyclase without receptor stimulation.

Effect of Amiloride on Alveolar Liquid Clearance

To investigate whether the amiloride-sensitive fraction of alveolar liquid clearance was altered after intestinal ischemia-reperfusion, anesthetized ventilated rats (control animals and animals subjected to intestinal ischemia-reperfusion) were instilled with the 5% albumin solution containing amiloride (10⁻³ M). The addition of amiloride inhibited alveolar liquid clearance to a similar degree in control and intestinal ischemia-reperfusion rats (Fig. 3, Table 1).

Effect of Neutralizing TNF-α MAbs on Alveolar Liquid Clearance After Intestinal Ischemia-Reperfusion

The effect of TNF-α on alveolar liquid clearance after intestinal ischemia-reperfusion was assessed by administration of a neutralizing anti-TNF-α MAb 30 min before the induction of intestinal ischemia. Administration of the anti-TNF-α MAb before induction of intestinal ischemia significantly reduced the increased alveolar liquid clearance after intestinal ischemia-reperfusion (P < 0.05; Fig. 4, Table 1). Alveolar liquid clearance after TNF-α inhibition was not different from that in sham-operated control rats (Fig. 4, Table 1).

Endothelial Permeability to Protein and Extravascular Lung Water

Extravascular accumulation of RITC-dextran in the lungs, determined as described in Endothelial Permeability to Protein and expressed as extravascular plasma equivalents, was used as a measure of endothelial protein leakage. Extravascular plasma equivalents did not differ between control rats and rats subjected to intestinal ischemia-reperfusion (Fig. 5, Table 2). Furthermore, propranolol did not affect the extravascular plasma equivalent accumulation in either control or intestinal ischemia-reperfusion rats (Table 2).

Administration of an anti-TNF-α MAb before the induction of the intestinal ischemia led to a significant...

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Fig. 2. Final-to-instilled protein concentration ratio over 45 min in sham-operated rats instilled with 5% albumin solution, rats subjected to intestinal I/R instilled with 5% albumin solution, sham-operated rats instilled with 5% albumin containing 10⁻⁴ M propranolol, and rats subjected to intestinal I/R instilled with 5% albumin containing 10⁻⁴ M propranolol. Values are means ± SD. Instillation of propranolol did not block the increase in alveolar liquid clearance seen after intestinal I/R. Thus endogenous catecholamine stimulation is not a principal mechanism. *Significant difference compared with control and control plus propranolol, P < 0.05 (by ANOVA).

Fig. 3. Final-to-instilled protein concentration ratio over 45 min in sham-operated rats instilled with 5% albumin solution, rats subjected to intestinal I/R instilled with 5% albumin solution, sham-operated rats instilled with 5% albumin containing 10⁻³ M amiloride, and rats subjected to intestinal I/R instilled with 5% albumin containing 10⁻³ M amiloride. Values are means ± SD. Instillation of amiloride inhibited similar fractions of alveolar liquid clearance in control and intestinal I/R rats. Significant difference (P < 0.05) compared with: *control and control plus amiloride; †I/R (both by ANOVA).
increase \( (P < 0.05) \) in extravascular plasma equivalent accumulation compared with that in rats subjected to intestinal ischemia-reperfusion alone and to sham-operated control rats (Fig. 5, Table 2).

Protein influx (measured by the vascular tracer RITC-dextran) from the plasma to the air spaces in rats subjected to intestinal ischemia-reperfusion compared with that in sham-operated control rats was similar in both groups (Table 2). Propranolol did not affect the protein influx in either sham-operated control rats or intestinal ischemia-reperfusion rats (Table 2). However, the alveolar-to-plasma concentration ratio of RITC-dextran was increased in rats subjected to intestinal ischemia-reperfusion after inhibition of TNF-\( \alpha \) with the MAb compared with that in the sham-operated rats as well as in the rats subjected to intestinal ischemia-reperfusion alone, although significance was not reached (Table 2).

Extravascular lung water was measured in rats subjected to intestinal ischemia-reperfusion and in sham-operated control rats without instillation of the alveolar test solution. Extravascular lung water was similar in the rats subjected to intestinal ischemia-reperfusion (4.5 \( \pm \) 0.4 g water/g dry lung; \( n = 4 \)) and in the sham-operated control rats (4.5 \( \pm \) 0.2 g water/g dry lung; \( n = 4 \)).

### Plasma Levels of TNF-\( \alpha \) After Intestinal Ischemia-Reperfusion

The plasma TNF-\( \alpha \) levels increased progressively over 60 min after initiation of intestinal reperfusion (Fig. 6). No change in TNF-\( \alpha \) levels was observed during the ischemic phase in the intestine.

### Table 2. Protein influx from plasma into alveolar spaces in rats subjected to sham operation or I/R

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Alveolar-to-Plasma Concentration, ratio of RITC-dextran</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0.06 ( \pm ) 0.04</td>
</tr>
<tr>
<td>I/R</td>
<td>9</td>
<td>0.04 ( \pm ) 0.02</td>
</tr>
<tr>
<td>I/R + TNF-( \alpha )  MAb</td>
<td>6</td>
<td>0.10 ( \pm ) 0.10</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SD; \( n \), no. of rats. RITC, rhodamine isothiocyanate.
In the samples obtained from the rats at the end of the experiments, TNF-\(\alpha\) plasma levels were increased in the intestinal ischemia-reperfusion group (Fig. 6, inset). No TNF-\(\alpha\) was seen in the sham-operated control rats. Administration of the neutralizing anti-TNF-\(\alpha\) MAb inhibited the intestinal ischemia-reperfusion-induced TNF-\(\alpha\) generation completely.

Effects on Systemic Blood Pressure and Peak Airway Pressure From Intestinal I\(\text{S}\)chemia-Re\(\text{P}\)erfusion

There were no significant differences in systemic arterial blood pressure expressed as mean arterial blood pressure, although there was a trend that the rats subjected to intestinal ischemia-reperfusion had a lower systemic blood pressure (control: 79 ± 30 mmHg, \(n = 11\); intestinal ischemia-reperfusion: 59 ± 18 mmHg, \(n = 11\); intestinal ischemia-reperfusion plus TNF-\(\alpha\) MAb: 64 ± 33 mmHg, \(n = 5\)). Peak airway pressures were the same in all experimental groups.

DISCUSSION

An intact barrier between the alveolar space and the vascular compartment is of critical importance for normal lung function. Substantial progress has been made in understanding the pathways and mechanisms regulating the clearance of protein and fluid across the alveolar and distal airway epithelia in the uninjured lung (reviewed in Ref. 20). The barrier properties may, however, change during pathological conditions such as acute lung injury and acute respiratory distress syndrome and result in severe pulmonary dysfunction. Increased endothelial permeability to protein has long been a known hallmark of acute lung injury, but it was not until recently that the alveolar and distal air space epithelial barrier has been thoroughly studied. A clinical study has shown that patients who were able to reabsorb alveolar edema fluid after acute lung injury had a better recovery from respiratory failure and a lower mortality compared with patients who were unable to reabsorb alveolar edema fluid (22).

Intestinal ischemia-reperfusion may lead to acute lung injury and acute respiratory distress syndrome characterized by increased pulmonary endothelial leakage and accumulation of inflammatory cells (15, 29). The barrier damage may be followed by an increased transvascular plasma leakage and fluid-filled alveolar spaces. In the present study, we investigated whether the injury to the lung endothelium and epithelium in the early phase of intestinal ischemia-reperfusion would be severe enough for alveolar flooding and/or interstitial pulmonary edema to occur. The study demonstrated that intestinal ischemia-reperfusion did not result in significant extravascular plasma accumulation in the alveolar spaces of the lung. Also, there seemed to be little accumulation of plasma in the interstitial spaces of the lungs. This finding was supported by the fact that there was no difference in extravascular lung water between rats subjected to intestinal ischemia-reperfusion and sham-operated control rats. This study, as well as a study by Khimenko et al. (14), demonstrated that alveolar fluid absorptive mechanisms were operational after both local lung ischemia-reperfusion and injury from remote ischemia-reperfusion such as intestinal ischemia-reperfusion injury. Because fluid is actively absorbed as a result of sodium uptake, our hypothesis was that sodium uptake was stimulated and that this resulted in an increased fluid absorption that counteracted an increased extravasation of fluid into the alveolar spaces. Our results show, in fact, that intestinal ischemia-reperfusion stimulates alveolar fluid clearance. Thus this would suggest that stimulation of alveolar fluid clearance may act as a first line of defense against alveolar flooding.

A variety of endogenous factors may be responsible for the stimulated alveolar liquid clearance after intestinal ischemia-reperfusion. Recent studies have suggested endogenous release of catecholamines, especially epinephrine, as an important mechanism for the stimulation of alveolar liquid clearance under normal physiological conditions (7) as well as under pathological conditions (16, 27). Finley et al. (7) showed that stimulation of alveolar liquid clearance for removal of fetal lung fluid in newborn guinea pigs in preparation for air breathing depended on elevated plasma epinephrine levels. Under pathological conditions, it has been demonstrated that the increased plasma epinephrine concentrations seen after septic shock in rats (27), as well as in a model of neurogenic pulmonary edema in dogs (16), stimulated alveolar liquid clearance. We therefore hypothesized that intestinal ischemia-reperfusion would lead to an increased release of endogenous catecholamines produced during the ischemic phase, in turn leading to \(\beta\)-adrenergic stimulation of alveolar fluid clearance during the reperfusion phase. We consequently carried out studies using the \(\beta\)-adrenergic antagonist propranolol to investigate the role of \(\beta\)-adrenergic-receptor stimulation and endogenous catecholamine release. We also investigated whether intracellular cAMP levels in lung tissue increased because studies (7, 8) have shown that \(\beta\)-adrenergic stimulation leads to elevations in intracellular cAMP that are associated with increased liquid clearance from the lung. However, contradictory to our hypothesis, we found that administration of the \(\beta\)-adrenergic antagonist propranolol was without effect on the stimulated alveolar liquid clearance after intestinal ischemia-reperfusion, indicating that the increased alveolar liquid clearance was not a result of epinephrine stimulation. This was further confirmed by our cAMP studies in which there was no stimulation of intracellular cAMP from intestinal ischemia-reperfusion. Therefore, it is unlikely that the stimulation of alveolar liquid clearance was secondary to increased plasma epinephrine levels and \(\beta\)-adrenergic stimulation.

Because the increase in alveolar liquid clearance was not mediated by the \(\beta\)-adrenergic system and because TNF-\(\alpha\) has been demonstrated to stimulate alveolar liquid clearance in rats after pneumonia (28), we hypothesized that TNF-\(\alpha\) could be involved. It has previously been shown that intestinal ischemia followed by reperfusion leads to increased intestinal
implies that the TNF-α-stimulated alveolar liquid clearance and edema formation by initially accelerating fluid transport out of the alveolar compartments.

We also investigated whether TNF-α-stimulated alveolar liquid clearance by increasing intracellular cAMP levels but found no such effect. This suggests that stimulation of alveolar liquid clearance during intestinal ischemia-reperfusion is via a pathway different from adenylate cyclase, which has previously been demonstrated to be one main intracellular pathway for stimulating liquid removal from the lung (7, 8). Therefore, although not tested in this study, the results suggest a direct action by the TNF-α receptor in stimulating alveolar liquid clearance. In fact, preliminary data from Jayr et al. (12) suggest that direct binding of TNF-α to its receptor in A549 pulmonary epithelial cells is directly linked with an increased capacity to transport sodium. However, in neither that study nor our investigation has the intracellular signaling pathway been definitively identified.

This is somewhat in contradiction to findings described by other authors, although one should bear in mind that anti-TNF-α therapy was shown to be inefficient in clinical trials on septic patients (1) and that treatment of septic rats with human recombinant TNF-α improved mortality and other parameters (2). Sorkine et al. (32) reported an increased leakage of radiolabeled albumin from the blood to the alveolar spaces after intestinal ischemia-reperfusion and that blocking of TNF-α reduces the lung injury. Caty et al. (5) also showed that intestinal ischemia-reperfusion induced a pulmonary microvascular injury that was prevented by pretreating the rats with anti-TNF-α antibodies. One explanation may be the different time periods of ischemia as well as the total study times used in the different investigations. In the study by Caty et al. (5), the duration of ischemia was 120 min compared with 45 min used in our model. At these longer study times, the injury may become too severe to the endothelium and epithelium and an increased absorption of fluid is not enough to maintain dry alveolar spaces. Also, if the epithelium and endothelium become severely injured and large holes open up in the barrier, the sodium gradient necessary for transepithelial fluid absorption cannot form. As a result, alveolar and interstitial flooding may occur.

Alveolar liquid clearance depends, in part, on amiloride-sensitive pathways (7, 23). Intra-alveolar amiloride inhibited 40% of the alveolar liquid clearance after intestinal ischemia-reperfusion as well as in the sham-operated animals in the present study, which is similar to what has previously been reported (11, 14, 23). This indicates that intestinal ischemia-reperfusion increases alveolar liquid clearance by stimulation of both amiloride-sensitive and amiloride-insensitive pathways. In fact, recent data (6, 30) suggest the existence of a rod-type cyclic nucleotide-gated cation channel in the alveolar epithelium that may be involved in fluid movement in the lung.

In conclusion, we found that intestinal ischemia-reperfusion in rats leads to stimulation of alveolar liquid clearance and that this stimulation is mediated, at least in part, by a TNF-α-dependent mechanism. Once TNF-α is eliminated, the alveolar epithelial-
endothelial barrier cannot withstand the pressure from
the circulation and plasma enters the extravascular
compartments in the lung. Our results therefore sug-

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