ACUTE RESPIRATORY distress syndrome (ARDS) is characterized by the leakage of protein and neutrophil-rich edema fluid into the lung interstitial and alveolar spaces (5). ARDS is a common result of several types of systemic inflammatory insults, including intestinal ischemia-reperfusion (I-R), that promote circulating neutrophil activation, lung neutrophil retention, and neutrophil-mediated pulmonary microvascular injury (8, 26, 35). When activated, neutrophils release several proteases (13, 15, 16, 18, 20, 25, 27, 28, 30–33, 37, 38), especially the serine protease elastase, which degrades proteoglycans in the glycocalyx (1) and components of the endothelial basement membrane (31, 32). The basement membrane and glycocalyx represent important functional components of the vascular barrier that limit fluid and solute exchange (7) and may also restrict granulocyte extravasation (38).

In addition to endothelial basement membrane and proteoglycan components, which limit exchange, the endothelial vascular barrier is maintained and regulated by junctional proteins termed “cadherins” (2, 19).

Neutrophil elastase promotes lung microvascular injury and proteolysis of endothelial cadherins

D. CARDEN, F. XIAO, Candace Moak, Bradley H. Willis, Sherry Robinson-Jackson, and Steve Alexander. Neutrophil elastase promotes lung microvascular injury and proteolysis of endothelial cadherins. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H385–H392, 1998.—Intestinal ischemia-reperfusion (I-R) is associated with lung injury and the acute respiratory distress syndrome. The hypothesis of this study was that intestinal I-R activates circulating neutrophils to promote elastase-mediated lung injury. Isolated rat lungs were perfused with blood or plasma obtained after intestinal I-R, and lung neutrophil retention and injury and bronchoalveolar lavage (BAL) elastase were measured. Perfusion with I-R caused lung neutrophil accumulation and injury and increased BAL elastase. These effects were attenuated by the elastase inhibitor L-658758. Interference with neutrophil adherence before gut reperfusion blocked BAL elastase accumulation. The role of endothelial junction proteins (cadherins) in I-R-elicited lung damage was also evaluated. Activated human neutrophils proteolyzed cadherins in human umbilical vein endothelial cells. Furthermore, plasma of patients with acute respiratory distress syndrome contained soluble cadherin fragments. The results of this study suggest that the elastase released by systemically activated neutrophils contributes to lung neutrophil accumulation and pulmonary microvascular injury. Elastase-mediated proteolysis of endothelial cell cadherins may represent the mechanism through which lung microvascular integrity is disrupted after intestinal I-R.

Acute respiratory distress syndrome; polymorphonuclear leukocyte

MATERIALS AND METHODS

Surgical Preparation

The animal experiments were approved in advance by the Louisiana State University Medical Center Animal Care and Use Committee. At the end of each experiment the deeply anesthetized rat was euthanized by thoracotomy and exsanguination. The procedures used to obtain human umbilical vein endothelial cells (HUVEC), polymorphonuclear leukocytes, and ARDS or control plasma were approved by the Louisiana State University Medical Center Institutional Review Board for Human Research.

Adult male Sprague-Dawley rats weighing 250–350 g were anesthetized with ketamine (100 mg/kg) and xylazine (7 mg/kg) intraperitoneally, then the trachea was intubated, the carotid artery was cannulated, and a ventral midline celotomy was performed. Anesthesia was maintained throughout the experimental protocol with supplemental ketamine and xylazine administered intraperitoneally. The superior mesenteric artery was completely occluded in blood or plasma donor animals with a noncrushing microvascular clamp, 5 ml of sterile saline were instilled intraperitoneally, and the incision was closed. After 120 min of ischemia, the vascular clamp was removed, the intestine was allowed to reperfuse for 20 min, and the animal was exsanguinated. Body temperature was maintained at 37 ± 0.5°C throughout the experimental protocol.

The animal whose heart and lungs were harvested for permeability determination was anesthetized with ketamine (100 mg/kg) and xylazine (7 mg/kg) intraperitoneally, then the trachea was intubated. The thoracic cavity was incised along both midaxillary lines, 500 U of heparin sodium were injected into the right ventricle, cannulas were placed...
into the pulmonary artery and the left atrium, and the heart and lungs were removed en bloc (7, 23). The isolated lungs were suspended by the trachea from a Grass Instruments force transducer in a warmed, humidified chamber. The lungs were immediately perfused with freshly obtained blood or plasma from three donor animals in an extracorporeal perfusion system by a constant-flow pump. Side ports to the pulmonary artery and left ventricle were connected to pres- sure transducers for continuous measurement of pulmonary arterial (Ppa) and pulmonary venous (Ppv) pressures, respectively. Ppa was maintained at 16 cmH2O by adjusting the flow rate of the perfusion reservoir, and Ppv was maintained at 4 cmH2O by adjusting the height of the blood reservoir. The lungs were ventilated with 2 cmH2O positive end-expiratory pressure to maintain the lungs inflated under zone 3 conditions (Ppa > Ppv > alveolar pressure).

Pulmonary Capillary Pressure

Pulmonary capillary pressure (Ppc) was measured to distinguish the fluid filtration (weight gain) that occurs secondary to changes in capillary pressure from that which is secondary to changes in microvascular permeability. Ppc was estimated using the double-occlusion technique, in which the arterial inflow and venous outflow lines are simultaneously occluded and the equilibrium Ppa and Ppv are recorded. This equilibrium pressure has been shown to correlate well with isogravi- metric measurements of Ppc.

Capillary Filtration Coefficient

The capillary filtration coefficient (Kf,c) is a specific and sensitive index of pulmonary microvascular permeability. After a 17-min isogravimetric period, Ppv was increased by 8 cmH2O and the change in lung weight was recorded. The rate of weight gain (Δwt/Δt) during the 6- to 17-min interval was analyzed using the linear regression of the log10-transformed rates of weight changes per minute, and the initial rate of weight gain was calculated by extrapolating Δwt/Δt to time 0. Kf,c was calculated by dividing Δwt/Δt at time 0 by the change in Ppv observed after elevation of venous outflow pressure, normalized to the initial wet lung weight, and expressed as milliliters per minute per centimeter H2O per 100 g of lung tissue.

Tissue Myeloperoxidase Activity

Myeloperoxidase (MPO) activity, a sensitive index of tissue neutrophil content, was determined in the entire left lobe of the isolated, perfused lung or in the left lobe of an excised, nonischemic lung that was not subjected to the isolation procedure (n = 6). Lung MPO content was determined by a modification of the method of Grisham et al. (12) using the peroxidase-catalyzed, H2O2-dependent oxidation of tetramethylbenzidine (TMB) as a measure of enzymatic activity. TMB oxidation was quantified by measuring the change in absorbance per minute at 655 nm and 37°C. One unit of MPO activity was defined as the amount of enzyme necessary to produce a change in absorbance per minute of 1.0.

Bronchoalveolar Lavage Elastase Activity

Bronchoalveolar lavage (BAL) fluid was obtained by infusing 1 ml of normal saline into the right lower lobe of the isolated perfused lung. Total BAL fluid elastase activity was determined by measuring the rate of p-nitroaniline formation from succinyl-L-Ala-Ala-L-Ala-p-nitroanilide (Calbiochem, San Diego, CA; 10 mM dissolved in N-methylpyrrolidone) spectrophotometrically at 410 nm. The reaction was performed in the presence of 1 M Tris buffer (pH 8.0) and terminated with 25 µl of glacial acetic acid.

To determine the contribution of intravascular neutrophils to BAL elastase accumulation, a separate group of animals (n = 6) was treated with a monoclonal antibody directed against the neutrophil adhesion molecule CD11/CD18 (CL26, 100 µg/animal) (35) before gut reperfusion and BAL elastase determination.

Experimental Protocols

Isolated rat lungs were perfused with nonischemic (control) blood (n = 6) or blood (n = 6) obtained after intestinal I-R. In a separate group of animals the specific elastase inhibitor L-658758 (Merck Sharpe and Dohme, Rahway, NJ) was added to the perfusion reservoir of the isolated, perfused lung. L-658758, a cephalosporin-based β-lactam, is a highly specific and irreversible elastase inhibitor (9). L-658758 inhibits the formation of the neutrophil elastase-α,α'-phenylenediamine inhibitor complex with an IC50 of 38 µM (22). The second-order rate constant (kinc/kd, where kinc is rate of inactivation and kd is inactivation constant) for inhibition of rat neutrophil elastase by L-658758 is 44,000 M−1·s−1·(11). Compared with the competitive trifluoromethylketone elastase inhibitor ICI-200880 and the reversible, noncompetitive elastase inhibitor Declaben, L-658758 appears to be unique in its ability to inhibit neutrophil elastase and the elastase-α,α'-phenylenediamine inhibitor complex in the biological milieu of blood (22).

Cell Culture and Treatment Protocols

HUVEC were harvested from umbilical cords by 0.25% collagenase digestion. The cells were cultured in endothelial cell growth medium (Clonetics) supplemented with 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), thymidine (2.4 mg/l; Sigma Chemical, St. Louis, MO), gluta- mine (230 mg/l; Gibco Laboratories, Gaithersburg, MD), heparin sodium (10 IU/ml; Sigma Chemical), and 100 IU/ml penicillin and 100 mg/ml streptomycin-amphotericin B with endothelial cell growth factor (80 µg/ml; Biomedical Technolo- gies, Stoughton, MA). All other tissue culture reagents were obtained from Gibco Laboratories. The cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO2 and expanded by trypsinization. HUVEC at passages 1 and 2 were seeded into fibronectin-coated (10 µg/ml) six- well tissue culture plates (Gibco Laboratories) and used when confluent (~3 days). Cells were identified as endothelial cells by cobblestone morphology, labeling with Dil-acetylated-low-density lipoprotein (Biomedical Technologies), and staining with anti-human factor VIII (Calbiochem).

Neutrophil-Mediated Proteolysis of VE-Cadherin

Neutrophil isolation. Human polymorphonuclear leukocytes were isolated from venous blood of healthy adults by use of standard dextran sedimentation and gradient separation on Histopaque 1077 (Sigma Chemical) (36). This procedure yields a 95–98% viable and 98% pure polymorphonuclear population. Experimental treatments (n = 3) were performed at confluency. In control experiments, Hanks’ balanced salt solution was placed onto the endothelial monolayers. In experiments in which neutrophils were used, the cells were added to monolayers at a neutrophil-to-endothelial cell ratio of 10:1. Neutrophils were activated with 10−5 M N-formylme- thionyl-leucyl-phenylalanine after being primed for 30 min with 1 µg/ml cytochalasin D. In experiments using elastase inhibitors, 10−4 M L-658758, 2.5 µg of α1-antichymotrypsin
ELASTASE-MEDIATED LUNG INJURY AND CADHERIN PROTEOLYSIS

ELISA for Soluble VE-Cadherin in Plasma Samples

To quantitate soluble VE-cadherin in plasma from control subjects (n = 5) and patients with ARDS (n = 6), an indirect ELISA was employed. ARDS patients met the American-European Consensus Conference definition of ARDS, including the presence of an arterial Po2-to-inspired O2 fraction of ≤200 mmHg (regardless of positive end-expiratory pressure), radiographically identified bilateral pulmonary infiltrates, and the absence of clinical or radiographic evidence of left heart failure. Heparinized plasma (200 µl) from healthy volunteers or patients with clinically defined ARDS was placed in a 96-well binding assay plate (Covalink, Bethesda, MD) for 30 min. Samples were removed, blocked with 1% Blotto in PBS, and reacted with anti-VE-cadherin antibody (1:1,000; clone BV9, Hemeris). Plates were washed three times with PBS plus 0.1% Blotto and reacted with secondary horseradish peroxidase-conjugated antibody (1:10,000) for 30 min. ELISA plates were incubated in TMB-H2O2 to produce a colorimetric reaction, and the reaction was terminated with 75 µl of 8 N sulfuric acid.

The ELISA for VE-cadherin is based on the high level of specificity of the BV9 antibody for human vascular endothelial cadherin (19). This antibody is species and protein selective and will not react with VE-cadherin from other species. This ELISA method is an effective means for qualitatively analyzing the amount of VE-cadherin in protein samples (14).

Statistical Analysis

Permeability, MPO, and BAL elastase data were analyzed by comparison of means with use of a one-way ANOVA. An unpaired Student's t-test was performed for evaluation of significant differences. Gels were analyzed using one-way ANOVA with Bonferroni posttesting. Absorbance values for the ELISA were compared statistically by unpaired Student's t-test. Significance was defined as P < 0.05. Values are means ± SE.

RESULTS

Effect of Intestinal I-R on Lung Neutrophil Retention (MPO Activity)

Compared with nonischemic (control) surgery, intestinal I-R (I-R blood) produced a significant increase in lung neutrophil retention (Fig. 1). Administration of the elastase inhibitor (L-658758 + I-R blood) significantly reduced the lung neutrophil retention elicited by intestinal I-R. As might be predicted, perfusion of lungs with only the plasma obtained after intestinal injury (I-R plasma) did not result in lung neutrophil accumulation. *P < 0.05 vs. control; #P < 0.05 vs. I-R.

Effect of Intestinal I-R on Lung Microvascular Permeability (Kf,c)

Compared with nonischemic (control) surgery, intestinal I-R (I-R blood) significantly increased lung microvascular permeability (Fig. 2). Administration of the elastase inhibitor (L-658758 + I-R blood) prevented the lung injury elicited by gut I-R. Perfusion of lungs with plasma obtained from animals subjected to intestinal I-R (I-R plasma) did not cause lung injury.

Effect of Intestinal I-R on BAL Elastase Content

Compared with nonischemic (control) surgery, intestinal I-R (I-R blood) resulted in a significant increase in total BAL fluid elastase content. Fig. 3). BAL fluid of lungs treated with the elastase inhibitor (L-658758 + I-R blood) or perfused with I-R plasma (I-R plasma) did not contain significant elastase activity.
Administration of the monoclonal antibody directed against the neutrophil adhesion molecule CD18 (CL26) blocked the accumulation of BAL elastase. These results suggest that the adherence of circulating neutrophils to pulmonary endothelium facilitates elastase release into the BAL fluid.

Activated Neutrophils Proteolyze VE-Cadherin

The amount of the intact (141 kDa) VE-cadherin contained in HUVEC was significantly reduced by exposure to activated neutrophils (Fig. 4), indicating that leukocytes cause a rapid proteolysis of endothelial cell VE-cadherin. This degradation of VE-cadherin by activated neutrophils was attenuated by administration of the elastase inhibitor L-658758, but not by α1-antichymotrypsin. FCS (2%) or BSA (0.5%) did not prevent neutrophil-mediated cadherin proteolysis. The dose-response effect of 0.25, 0.5, 1, and 2% FCS and 0.1, 0.5, and 1% BSA on VE-cadherin proteolysis was also determined. Regardless of the applied dose, neither FCS nor BSA blocked cadherin proteolysis (data not shown).

Purified Human Neutrophil Elastase Degrades VE-Cadherin

Figure 5 indicates that HUVEC exposed to purified human neutrophil elastase (1 µg/ml) exhibited proteolysis of the normal 141-kDa HUVEC VE-cadherin, resulting in the generation of 92- and 50-kDa fragments. These results are similar to those produced by activated neutrophils (Fig. 4).

ELISA for Soluble VE-Cadherins in ARDS and Control Patient Samples

ELISA of soluble immunoreactive VE-cadherin indicates a significant increase in soluble VE-cadherin in the plasma of ARDS patients compared with normal controls (P < 0.05; Fig. 6). These results indicate that, in ARDS, cleavage of endothelial VE-cadherin occurs, yielding a soluble form of VE-cadherin that can be detected in plasma.

DISCUSSION

Previous studies have demonstrated that the systemic inflammation initiated by intestinal I-R often produces a neutrophil-mediated acute lung injury (8, 26, 35). The results of the current investigation are unique, in that they demonstrate that the pulmonary microvascular damage associated with gut I-R is produced by neutrophil elastase. Importantly, the predominant source of elastase in this model is activated neutrophils from the systemic circulation, rather than resident lung leukocytes, since whole blood, but not plasma, obtained after gut I-R promotes pulmonary microvascular injury and BAL fluid elastase accumulation. This study also indicates that an important mechanism by which neutrophil elastase disrupts microvascular barrier integrity may involve the proteolysis of VE-cadherin.
interendothelial cell junctional proteins termed cadherins. There is a large body of evidence implicating neutrophils in the microvascular injury incurred on reperfusion of ischemic tissues and in the lung injury elicited by systemic inflammation (6–8, 10–13, 23, 26, 33, 35, 36, 38, 39). ARDS is an important clinical syndrome that often follows systemic inflammation and is characterized by lung neutrophil retention and neutrophil-mediated lung microvascular injury. Evidence in vivo and in vitro suggests that elastase is likely the principal injurious enzyme released by neutrophils in ARDS (15, 20). For example, BAL fluid of ARDS patients contains elevated amounts of elastase (20), and in vitro studies show that the neutrophil-mediated increase in vascular permeability observed in ARDS is the direct result of elastase (15).

Evidence supports a major role for elastase in the disruption of the microvascular barrier in other models of lung injury as well. Elastase has been implicated in the development of acute edematous lung injury elicited by oxidants (6), hyperoxia (24), and hindlimb ischemia (33). In addition, endothelial cells exposed to anoxia-reoxygenation provoke neutrophils to release elastase (13), which causes neutrophil-mediated injury to microvascular endothelial cells (27). The results presented in this report provide additional support for a critical role for elastase in the loss of integrity of the microvascular barrier, in that the specific elastase inhibitor L-658758 attenuates the increased pulmonary microvascular permeability elicited by intestinal I-R.

Although abundant evidence implicates circulating neutrophils as the main source of elastase-mediated lung damage (8, 26, 35), it is also possible that resident lung neutrophils or alveolar macrophages are activated by I-R to release elastase and elicit lung microvascular dysfunction (23). For example, it has been shown that administration of leukotriene B₄, a proinflammatory, chemotactic mediator released systemically after gut I-R (21, 39), to the isolated, perfused rabbit lung increases perfusate neutrophil elastase content and microvascular permeability. These effects are blocked by a competitive elastase inhibitor (37). Thus stimulated alveolar macrophages or resident lung neutrophils could cause pulmonary injury after systemic inflammation. To address this possibility, we perfused isolated lungs with plasma obtained after intestinal I-R. Lungs perfused with I-R plasma did not exhibit lung injury or increased BAL elastase content. Furthermore, inhibition of adherence of intravascular neutrophils to lung endothelium prevented the accumulation of BAL elastase (Fig. 3). We previously reported that antibodies that block neutrophil or endothelial adher-
Adhesive determinants prevent lung injury in this model (35). Taken together, our results suggest that the adherence of circulating neutrophils, rather than the resident population of lung leukocytes, to endothelium facilitates elastase release into the BAL fluid and contributes to elastase-mediated lung injury.

Although substantial evidence supports neutrophil-derived elastase in lung microvascular dysfunction, plasma and interstitial fluid contain proteinase inhibitors, such as α1-proteinase inhibitor, that normally protect tissue from the unregulated action of neutrophil elastase (24, 31, 32). However, it has been reported that plasma and serum as well as purified α1-proteinase inhibitor do not prevent elastase-mediated injury to human microvascular endothelial cells in vitro (27).

Additionally, α1-proteinase inhibitor is inactivated by neutrophil-derived oxidants (24, 31, 32), which might allow elastase to degrade the subendothelial matrix in the oxidant-rich microenvironment between adherent neutrophils and the underlying substrate. The observation that FCS, a potent source of neutrophils and the underlying substrate. The observation that FCS, a potent source of neutrophils and the underlying substrate. The observation that FCS, a potent source of neutrophils and the underlying substrate.

Because neutrophils can oxidatively inactivate α1-proteinase inhibitor, (24, 31, 32), the protective effects of the antiprotease can be overcome, and elastase-mediated injury can proceed unchecked. However, when elastase alone is added to monolayers (Fig. 5), oxidants are not available to inactivate α1-proteinase inhibitor, and the antiprotease can then effectively function as an elastase inhibitor. The ability of neutrophils to inactivate α1-proteinase inhibitor may also account for the extensive cadherin proteolysis mediated by neutrophils compared with elastase alone (Figs. 4 and 5). These results suggest that systemic inflammation may result in an oxidant-mediated loss of normal antiprotease defenses.

The targets through which neutrophil elastase disrupts microvascular integrity are not established. Because elastase is capable of degrading many components of the glycosaminoglycans and basement membrane, it is possible that the elastase-mediated injury to endothelial cells represents increased access of elastase to the subendothelial matrix (13). An alternate explanation for the ability of elastase to increase vascular permeability is that neutrophil elastase may hydrolyze cell-cell adhesion proteins such as cadherins and diminish vascular barrier. Cadherins are junctional proteins that maintain cell-cell adhesion and hence barrier in endothelial and epithelial cells (2, 19).

The observation that VE-cadherin is proteolized by activated neutrophils as well as by purified human neutrophil elastase (Figs. 4 and 5) suggests that neutrophils alter endothelial junctional integrity through elastase-mediated proteolysis of junctional proteins. The fact that soluble VE-cadherin is detectable in the plasma of patients with ARDS (Fig. 6) suggests that a potential mechanism of lung injury in ARDS may involve elastase-mediated proteolysis of cadherin junctional proteins. By degrading the components of the endothelial junction, elastase may reduce the vascular barrier to macromolecules. It would be ideal to also measure cadherin fragments in rat plasma samples after gut I-R in this model. However, no antibodies recognizing rat VE-cadherin are available.

The effects of neutrophil elastase in the microvasculature may involve more than simply enhanced permeability associated with the degradation of interendothelial junctional proteins, proteoglycans in the glycosaminoglycans, or glycoproteins in the basement membrane. For example, L-658758 reduces neutrophil adhesion, emigration (38), and retention (7) in postischemic tissue. On the basis of these observations, Zimmerman and Granger (38) suggested that neutrophils employ elastase to digest a route of passage from the vascular to the tissue compartments. Woodman et al. (34) also provided evidence that elastase contributes to tissue neutrophil accumulation by mechanisms that actually precede tissue emigration. These investigators demonstrated that elastase induces a dose-dependent upregulation of CD11/CD18 on neutrophils that is prevented by L-658758. Other studies report that proteinase inhibitors can inhibit N-formylmethionyl-leucyl-phenylalanine-induced neutrophil polarization and motility of neutrophils in vitro (4). These results indicate that neutrophil elastase may contribute to the directed migration of leukocytes into inflamed postcapillary venules in addition to contributing to the process of tissue emigration. Our results showing that L-685758 reduces lung neutrophil accumulation after gut I-R argue that leukocyte migration,
entrapment, and/or adhesion depends on the release of neutrophil elastase.

Although it is possible that the L-658758-mediated reduction in lung leukocyte accumulation contributes to diminished lung injury after gut I-R, it is also clear that lung leukocyte accumulation alone is not sufficient to elicit pulmonary microvascular injury (8, 35). For example, we have reported that inhibitors of neutrophil adherence prevent lung injury after intestinal I-R, yet have no effect on the marked increase in lung neutrophil content elicited by the gut insult (35). The lung neutrophil accumulation after gut I-R appears to be at least partially dependent on changes in neutrophil microvascular damage after systemic inflammation.

In summary, the results of this report demonstrate that systemic inflammation initiated by intestinal I-R is associated with lung neutrophil retention and disruption of the lung endothelial barrier by the action of neutrophil-derived elastase. Our data also suggest that elastase-mediated proteolysis of endothelial cadherins may represent a novel mechanism by which neutrophils disrupt the integrity of the microvascular barrier in ARDS.

This work was supported by National Institutes of Health Grants 2-P01-DK-4378506 and HL-47615 and the Biomedical Research Foundation of Northwest Louisiana.

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Received 11 August 1997; accepted in final form 16 April 1998.

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