Complement-mediated lung injury and neutrophil retention after intestinal ischemia-reperfusion

FENG XIAO, MICHAEL J. EPPIHIMER, BRADLEY H. WILLIS, AND DONNA L. CARDEN

Departments of Medicine and Physiology and Biophysics, Louisiana State University Medical Center, Shreveport, Louisiana 71130

Xiao, Feng, Michael J. Epiphimer, Bradley H. Willis, and Donna L. Carden. Complement-mediated lung injury and neutrophil retention after intestinal ischemia-reperfusion. J. Appl. Physiol. 82(5): 1459–1465, 1997.—Complement-mediated neutrophil activation appears to play an important role in ischemia-reperfusion (I/R) injury in a variety of tissues, including the heart, lung, and small bowel. The objective of this study was to determine whether inhibition of the alternative and classic complement cascades by administration of soluble complement receptor 1 (sCR1) prevents the increased neutrophil stiffness, lung neutrophil retention, and pulmonary microvascular injury elicited by a systemic inflammatory insult. Isolated lungs were perfused with blood obtained from animals subjected to 2 h of intestinal ischemia and 20 min of reperfusion (I/R) or control (nonischemic) surgery. Intestinal I/R resulted in a significant increase in neutrophil stiffness, lung neutrophil retention, and increased pulmonary microvascular permeability, effects that were prevented by administration of sCR1 before intestinal reperfusion. The results of this study suggest that I/R injury in the gut is a potent systemic inflammatory stimulus that induces complement-mediated neutrophil stiffness, lung neutrophil entrapment, and pulmonary microvascular dysfunction.

Complement activation; acute respiratory distress syndrome; pulmonary capillary filtration coefficient; neutrophil deformability

INTESTINAL ISCHEMIA, as a primary event or as an adaptive response to systemic hypotension, is a common clinical problem associated with the systemic inflammatory response syndrome. Lung neutrophil retention occurs as a consequence of systemic inflammation and results in increased lung microvascular permeability and the development of noncardiogenic pulmonary edema, commonly termed acute respiratory distress syndrome (ARDS) (2, 3, 17, 18).

Although systemic inflammation and ARDS are characterized by neutrophil entrapment in the lung, the mechanism by which these inflammatory cells are arrested in the pulmonary circulation and contribute to the disruption of lung microvascular barrier integrity is not clear. It is recognized, however, that enhanced neutrophil stiffness is an important determinant of granulocyte sequestration in the pulmonary microvasculature (13, 29). In addition, there is evidence that suggests that activation of the complement cascade contributes to the pulmonary microvascular dysfunction observed in ARDS (16, 22). For example, complement activation promotes lung neutrophil retention and pulmonary injury after gut ischemia (18) and in ARDS (16). There is also evidence that infusion of complement-activated plasma induces pulmonary microvascular dysfunction that mimics that observed in ARDS (5).

The purpose of this study was to assess the contribution of complement activation to the enhanced neutrophil stiffness, lung neutrophil retention, and pulmonary microvascular dysfunction elicited by intestinal ischemia-reperfusion (I/R). To achieve this objective, the effect of soluble complement receptor 1 (sCR1; generously provided by T Cell Sciences, Needham, MA), a potent and specific inhibitor of both the alternative and classic complement cascades, on neutrophil deformability, lung neutrophil retention, and pulmonary microvascular permeability was determined.

MATERIALS AND METHODS

The animal experiments described were approved in advance by the Institutional Animal Care and Use Committee. At the end of each experiment, the deeply anesthetized rat was killed by thoracotomy and exsanguination.

Preparation

Adult male Sprague-Dawley rats weighing 250–350 g were anesthetized with ketamine (50 mg/kg ip) and xylazine (7 mg/kg ip), after which the trachea was intubated, the carotid artery was cannulated, and a ventral midline celiotomy was performed. Anesthesia was maintained throughout the experimental protocol with supplemental intraperitoneally administered ketamine and xylazine. The superior mesenteric artery was completely occluded in three blood 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 and the intestine was allowed to reperfuse for 20 min. Body temperature was maintained at 37 ± 0.5°C throughout the experimental protocol.

The animal, the heart and lungs of which were harvested for permeability determination, was subjected to an identical protocol described above without celiotomy or gut I/R (Fig. 1). The thoracic cavity was incised along both midaxillary lines, 500 U 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. The isolated lungs were suspended by the trachea from a Grass Instruments force transducer in a warmed, humidified chamber. The lungs were perfused with freshly obtained whole donor blood in an extracorporeal perfusion system by a constant-flow pump. Side ports to the pulmonary artery and left ventricle were connected to pressure transducers for continuous measurement of pulmonary arterial (Ppa) and pulmonary venous (Ppv) pressures, respectively. Ppv was maintained at 4 cmH₂O by height adjustment 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 (Ppc)

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 by using the double-occlusion technique (26), 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 isogravimetric measurements of Ppc (26).

Capillary Filtration Coefficient (Kf,c)

The Kf,c is a specific and sensitive index of pulmonary microvascular permeability (1, 14). 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 by using the linear regression of the log10-transformed rates of weight changes per minute, and the initial rate of weight gain was calculated by dividing Δwt/Δt at time 0 by the change in Ppc (determined as described above) observed after elevation of venous outflow pressure, normalized to the initial wet lung weight and expressed as milliliters per minute per centimeters water per 100 g lung tissue.

Plasma Complement Activity

Total hemolytic complement activity was determined by the radial immunodiffusion technique (The Binding Site, Birmingham, UK). Serum samples obtained from animals subjected to control or intestinal I/R surgery in the presence or absence of sCR1 were placed in wells embedded in an agarose gel containing sensitized sheep erythrocytes. The diameter of lysis of sensitized erythrocytes is dependent on total serum complement activity.

Tissue Myeloperoxidase (MPO) Activity

MPO activity, a sensitive index of tissue neutrophil infiltration, was determined in the entire left lobe of the isolated, perfused lung by a modification of the method of Grisham et al. (15) by using the peroxidase-catalyzed, H2O2-dependent oxidation of tetramethylbenzidine as a measure of enzymatic activity. Tetramethylbenzidine 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.

Neutrophil Isolation

Whole, nonheparinized blood obtained from rats subjected to control or intestinal I/R surgery in the presence or absence of sCR1 was collected in EDTA, and neutrophil isolation was accomplished (NIM.2, Cardinal Associates, Santa Fe, NM) by using a sucrose polymer and sodium diatrizoate density gradient for neutrophil separation. After centrifugation at 200°C, the lower, neutrophil-rich band was collected and washed in phosphate-buffered saline (PBS), and the final cell count was assessed by hemacytometer and adjusted to 2,000/µl (4 x 10^5 in 200 µl). The cell suspension was filtered through a 12-µm-pore polycarbonate filter, placed on ice, and used immediately for assessment of neutrophil deformability.

Neutrophil Deformability

To assess the potential for neutrophil sequestration in the microvasculature in vivo, the filterability of neutrophil suspensions through 5-µm Nuclepore filters (Nuclepore, Pleasanton, CA), as described by Downey and Worthen (11) and modified by Eppihimer and Lipowski (13), was examined in vitro. With the use of this technique, the deformability of the circulating population of neutrophils was determined in terms of a statistical distribution of yield pressures required to dislodge...
a sample of neutrophils trapped within the pores of the filter (13).

Nuclepore filters (13-mm diameter, 11-µm thick, mean pore density 4 x 10^6 pores/cm²) were perfused by a syringe filled with cell-free PBS driven by an infusion pump. A 200-ml neutrophil suspension, isolated after control or intestinal I/R surgery in the presence or absence of sCR1, was introduced as a bolus proximal to the filter at an initial flow rate of 0.23 ml/min. The filter was perfused for 1–2 min, during which time the majority of neutrophils were distributed and trapped in the filter pores. The pressure drop across the filter in response to subsequent, incremental increases in flow of cell-free PBS (0.23–10.0 ml/min) was measured and recorded to within ±0.01 cmH₂O. Each stepwise increase in flow was maintained for a period of 1–2 min to ensure that all entrapped neutrophils characteristic of that perfusion pressure were expelled from the filter. The stepwise increases in flow rate resulted in disproportionately smaller increases in pressure drop across the filter as fractions of neutrophils were dislodged from the pores. The average deformability of the neutrophil suspension was determined as the mean of the distribution of yield pressures (Pyield).

White Blood Cell Counts

Aliquots of whole blood were obtained for peripheral white blood cell counts from blood donor animals after the surgical preparation described above and before lung perfusion. The blood samples were diluted in 3% acetic acid to lyse erythrocytes, and the nuclei of the leukocytes were stained with crystal violet (0.01%). The leukocytes were counted with a hemacytometer, and neutrophil counts were expressed as cells per cubic millimeter of whole blood.

Experimental Protocols

Group 1: Nonischemic, control surgery (control; n = 6). Isolated lungs, perfused with blood obtained from animals subjected to control surgery, were allowed to equilibrate and remain isogravimetric for 17 min. Ppa was maintained at 16 cmH₂O and Ppv at 4 cmH₂O. Determinations of Kf,c and Ppc were obtained, and the left lung was excised for MPO determination.

Group 2: Intestinal I/R (I/R; n = 6). Isolated lungs, perfused with blood obtained from animals subjected to intestinal I/R, were prepared surgically as described above. Determination of Kf,c, Ppc, and MPO were determined as for group 1 above.

Group 3: Intestinal I/R + sCR1 (I/R + sCR1; n = 5). Isolated lungs in this group were perfused with blood obtained from animals treated with sCR1 (20 mg/kg ia) 5 min before intestinal reperfusion. In addition, 0.286 ng/ml sCR1 was added to the perfusion reservoir of the isolated, perfused lung.

sCR1 is a potent inhibitor of both the classic and alternative pathways of complement activation (27). It binds to C3b and C4b, thus inhibiting the C3 and C5 convertases and also serves as a cofactor in factor I-mediated proteolysis of C3b and C4b. sCR1 prevents the release of the proinflammatory anaphylatoxins C3a and C5a and prevents the downstream assembly of the membrane attack complex (27).

Statistical Analysis

Data were analyzed by comparison of means by using a one-way analysis of variance. An unpaired Student's t-test was performed for evaluation of significant differences. Repeated measures was performed for evaluation of plasma complement activity, and paired analyses were used where appropriate. Significance was defined as P < 0.05. All analyses are expressed as means ± SE.

RESULTS

Pulmonary Microvascular Permeability

Pulmonary microvascular permeability (Fig. 2), as determined by Kf,c, was significantly increased in lungs perfused with blood obtained from animals subjected to intestinal I/R (0.130 ± 0.012 ml·min⁻¹·cmH₂O⁻¹·100 g lung wt⁻¹) compared with lungs perfused with control blood (0.082 ± 0.015 ml·min⁻¹·cmH₂O⁻¹·100 g lung wt⁻¹, P < 0.05). Administration of sCR1 before reperfusion prevented the lung injury elicited by gut I/R (0.080 ± 0.012 ml·min⁻¹·cmH₂O⁻¹·100 g lung wt⁻¹; P > 0.05 vs. control; P < 0.05 vs. I/R).

Serum Complement Activity

Figure 3 depicts the serum complement activity in animals subjected to control or intestinal I/R surgery. Compared with baseline values, serum complement activity was significantly decreased after 5 min of intestinal reperfusion (diameter of lysis = 6.5 ± 0.25 and 5.87 ± 0.2 mm, respectively; P < 0.05). The decrease (consumption) in total complement activity observed at 5 min of intestinal reperfusion was abolished in animals administrated sCR1 (diameter of lysis = 6.33 ± 0.17 mm). There was no significant difference in serum complement activity at any time in animals subjected to control surgery.

Lung Neutrophil Accumulation

Lung neutrophil content (Fig. 4), as determined by lung MPO activity, was significantly increased in lungs perfused with blood obtained from animals subjected to intestinal I/R compared with lungs perfused with control blood (11.34 ± 1.27 and 3.19 ± 0.63 U/g wet wt, respectively; P < 0.05). Administration of sCR1 before reperfusion attenuated the lung neutrophil retention elicited by gut I/R (6.18 ± 1.59 U/g wet wt; P > 0.05 vs. control; P < 0.05 vs. I/R).

Statistical Analysis

Data were analyzed by comparison of means by using a one-way analysis of variance. An unpaired Student's t-test was performed for evaluation of significant differences. Repeated measures was performed for evaluation of plasma complement activity, and paired analyses were used where appropriate. Significance was defined as P < 0.05. All analyses are expressed as means ± SE.
Neutrophil Deformability

There was no significant difference in the number of cells/200 µl in the final cell suspensions among groups (3.8 – 4.2 × 10^5/200 µl in control, 3.9 – 4.2 × 10^5/200 µl in I/R, and 4.0 – 4.2 × 10^5/200 µl in I/R + sCR1; P > 0.05). Hematocrits in the final cell suspensions were <2%, and the viability of neutrophils in all experimental groups, as determined by trypan blue exclusion, was >95%.

Intestinal I/R caused a significant increase in neutrophil stiffness compared with control surgery (Fig. 5; mean \( P_{\text{yield}} = 3.614 \pm 0.294 \) and \( 2.519 \pm 0.234 \) cmH_2O, respectively; \( P < 0.05 \)). Administration of sCR1 prevented the neutrophil stiffness induced by gut I/R (\( 2.791 \pm 0.181 \) cmH_2O; \( P > 0.05 \) vs. control; \( P < 0.05 \) vs. I/R). However, sCR1 had no effect on the baseline deformability of neutrophils isolated after control surgery (\( P_{\text{yield}} = 2.862 \pm 0.071 \) cmH_2O).

DISCUSSION

Intestinal I/R is a common clinical problem associated with the development of systemic inflammation, pulmonary microvascular dysfunction, and ARDS (2, 3, 17, 18). Pulmonary insufficiency has been reported in patients undergoing surgical revascularization for mesenteric arterial occlusive disease (17), and lung injury has been demonstrated experimentally after gut I/R (2, 3, 18). However, alterations in neutrophil deformability have not been directly measured after intestinal I/R, and the relationship among neutrophil stiffness, lung neutrophil accumulation, and lung injury after a systemic inflammatory insult has not been examined. The results of the present investigation are unique in that they provide direct evidence that complement-mediated increases in neutrophil stiffness occur early after intestinal reperfusion in vivo, are maintained for at least 20 min, and are associated with lung neutrophil accumulation and injury. The present model of systemic inflammation-elicted lung injury is also unique in that it demonstrates that intestinal I/R is a potent inflammatory stimulus that disrupts normal lung microvascular integrity. In the utilization of this model, the inflammatory signal in the intestine and the mechanism of systemic inflammation-induced microvascular injury in the lung can be individually examined. The fact that...
enhanced neutrophil stiffness, lung neutrophil sequestration, and lung injury are attenuated by inhibition of the alternative and classic complement cascades before reperfusion provides direct evidence that complement activation in the postischemic intestine is an early and critical component of the systemic inflammatory response syndrome.

Lung neutrophil retention occurs as a consequence of systemic inflammation (2, 3, 18) and is a consistent finding in studies of ARDS (16, 19, 20, 22). Although the systemic inflammatory response elicited by gut I/R is characterized by neutrophil retention in the lung (2, 3, 18), the mechanism by which these inflammatory cells become entrapped in the pulmonary microvasculature is not clear. However, intestinal I/R can induce systemic leukocytosis, raising the possibility that an increased number of circulating neutrophils account for increased lung neutrophil content. Our results indicate that leukocytosis alone is not sufficient to cause lung neutrophil retention because sCR1-treated animals had a greater number of circulating neutrophils than rats subjected to intestinal injury without sCR1. Despite increased circulating neutrophils, lungs perfused with sCR1-treated I/R blood retained significantly fewer neutrophils than lungs perfused with untreated, I/R blood. The increased number of circulating neutrophils in sCR1-treated animals is consistent with an inhibition of neutrophil retention in the lungs of these blood-donor animals as well as in the isolated, perfused normal lung. These results confirm the importance of complement activation in mediating lung neutrophil retention after intestinal I/R.

Additional evidence suggests that activation of the complement cascade contributes to neutrophil-endothelial cell adhesion both in vitro and in vivo. For example, endothelial cell deposition of the complement protein C3 generates rapid neutrophil adhesion mediated by the neutrophil glycoprotein CD11b/CD18 (21). Furthermore, inhibition of complement activation prevents the initial upregulation of neutrophil CD11b/CD18 and diminishes the early endothxin-induced accumulation of neutrophils in the liver (28). The fact that there is a correlation between bronchoalveolar lavage fluid C5a and chemotactic activity and the presence of lavage fluid neutrophils in patients with ARDS provides supportive evidence that complement activation contributes to lung neutrophil entrapment in this disorder (22). Direct evidence that complement activation mediates postischemic tissue neutrophil retention is provided by investigations in which inhibition of complement activation by administration of sCR1 diminishes reperfusion-induced neutrophil retention in the heart (27), lungs (present study), and intestine (18).

Complement activation not only contributes to neutrophil-endothelial cell adhesion but also may mediate endothelial cell injury. For example, infusion of complement-activated serum induces plasma leakage in mesenteric vessels within 20 min of administration (19). In addition, exposure of human umbilical vein endothelial cells to both activated complement and neutrophils results in significant endothelial cell injury, whereas exposure to either agent alone does not (23). Experimental and clinical evidence also suggest that complement-mediated lung neutrophil sequestration is associated with enhanced pulmonary microvascular permeability (5).

Although the mechanism by which complement activation contributes to endothelial cell injury is not defined, there is evidence that suggests that complement-activated neutrophils induce endothelial cell injury primarily by the generation of reactive oxygen metabolites (23). In addition, activated complement contributes to the initial, endotoxin-induced upregulation of CD11b/CD18, the neutrophil adhesion glycoprotein that is responsible for adherence-dependent reactive oxygen metabolite formation (25). These results suggest that the generation of oxidants by complement-stimulated neutrophils contributes to neutrophil-mediated endothelial cell dysfunction.

Although complement activation clearly contributes to neutrophil-endothelial adherence, it has been suggested that alterations in neutrophil stiffness rather than adherence per se are responsible for the physiological retention of neutrophils in the pulmonary microvasculature (6, 8, 29). In fact, the ability of neutrophils to invade the pulmonary vascular bed is inversely proportional to cell stiffness (8). The concept that leukocyte stiffness contributes to lung neutrophil retention is also supported by evidence in humans, in whom a correlation has been reported between lung neutrophil sequestration in vivo and neutrophil deformability in vitro (24).

Neutrophil deformability is particularly relevant to neutrophil retention in the microvasculature of the lung for several reasons. Neutrophils are normally 1,000 times less deformable than erythrocytes, and granulocytes become delayed with respect to erythrocytes in their passage through the microvasculature of the lung (4, 9). In addition, the mean diameter of neutrophils (7–8 mm) is larger than the mean diameter of pulmonary capillaries (5–5.5 mm) (9). Furthermore, the intravascular pressures in the pulmonary capillaries are one-tenth those in the systemic circulation, and the flow within the microvasculature of the lung is pulsatile, with periods of flow interspersed with intervals of no flow (12). Thus the effects of hydrodynamic dispersal forces and neutrophil deformability are important determinants of neutrophil retention in the pulmonary microcirculation (12).

Exposure of neutrophils to inflammatory stimuli results in a decrease in their deformability because of reorganization of the neutrophil cytoskeleton and net filamentous actin (F-actin) assembly (29). These changes diminish the ability of leukocytes to deform during capillary transit (13, 29) and cause neutrophil retention within capillary-sized pores in vitro and in the pulmonary microvasculature in vivo (29). A novel finding of the present study is that I/R injury in the gut is a sufficient inflammatory stimulus to elicit an increase in neutrophil stiffness and lung neutrophil entrapment. These effects are prevented by complement inhibition before intestinal reperfusion, suggesting that comple-
sCR1 attenuates lung injury after intestinal I/R

ment-mediated neutrophil stiffening contributes to lung neutrophil retention after gut I/R.

Although changes in neutrophil deformability and neutrophil-endothelial cell adhesion have independently been implicated in the entrapment of neutrophils in the lung, it is possible that the two events are actually interrelated (6, 10, 23). CD18-dependent adhesion may be enhanced by leukocyte stiffening, which facilitates close apposition and interaction between the leukocyte and the microvascular endothelium (10). In fact, the pulmonary margination of neutrophils elicited by infusion of complement-activated plasma has an initial phase (1–7 min after infusion) that depends on a change in neutrophil deformability and a later phase (>15 min after infusion) that is CD18 dependent (6). The results of the present study are consistent with the hypothesis that complement activation occurs early after intestinal I/R and elicits an increase in neutrophil stiffness at time intervals beyond the 7 min previously reported after infusion of complement-activated plasma (6). Furthermore, the enhanced neutrophil stiffness is associated with significant lung neutrophil retention that may facilitate a later, adhesion-dependent neutrophil accumulation.

Consistent with the hypothesis that prolonged gut reperfusion is associated with complement-independent lung neutrophil retention, Hill and colleagues (18) assessed lung neutrophil retention, Hill and colleagues (18) assessed lung neutrophil retention at 3 h rather than at 20 min of intestinal reperfusion and reported that sCR1 attenuates lung injury but not lung neutrophil sequestration after the gut insult. This finding raises the possibility that prolonged reperfusion leads to the release of inflammatory mediators other than activated complement that contribute to lung neutrophil retention. It is possible that neutrophil-endothelial cell adhesion, rather than altered neutrophil stiffness, is a critical determinant of lung neutrophil sequestration after 3 h of intestinal reperfusion. In addition, lung neutrophil retention was determined in the animal subjected to intestinal I/R in the Hill et al. study as opposed to a normal lung in the present investigation. Before reperfusion, prolonged instrumentation, anesthesia, or gut ischemia may promote lung neutrophil retention in the animal exposed to intestinal surgery, but these events would not affect neutrophil entrapment in the normal lung. Finally, the higher dose of sCR1 used in the present study (20 mg/kg) compared with the dose of sCR1 administered by Hill et al. (6–12 mg/kg) may have contributed to the attenuation of neutrophil stiffness and lung neutrophil retention observed in the present study. Importantly, sCR1 administered before intestinal reperfusion diminished the enhanced lung microvascular permeability observed after 20 min of intestinal reperfusion and after the prolonged gut reperfusion observed by Hill et al.

In conclusion, the results of this study suggest that intestinal I/R is associated with an increase in neutrophil stiffness and lung neutrophil retention as well as an increase in lung microvascular permeability. Inhibition of the alternative and classic complement cascade by administration of sCR1 before intestinal reperfusion prevented these manifestations of systemic inflammation elicited by gut I/R. Activation of the complement cascade appears to be an early event and important mediator of neutrophil retention and neutrophil-mediated pulmonary dysfunction elicited by intestinal I/R.

Address for reprint requests: D. L. Carden, Dept. of Internal Medicine, Louisiana State Univ. Medical Center, 1501 Kings Highway, Shreveport, LA 71130.

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