Role of neutrophils in ischemia-reperfusion-induced microvascular injury

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Department of Physiology, College of Medicine, University of South Alabama, Mobile, Alabama 36688; Department of Physiology and Biophysics, Louisiana State University Medical Center, Shreveport, Louisiana 71130; Pharmacia Experimental Medicine, LaJolla, California 92037; and Department of Medicine, University of Washington, Seattle, Washington 98104

HERNANDEZ, Lucrecia A., Matthew B. Grisham, Beverleigh Twohig, Karl E. Arfors, John M. Harlan, and D. Neil Granger. Role of neutrophils in ischemia-reperfusion-induced microvascular injury. Am. J. Physiol 253 (Heart Circ. Physiol. 22): H699-H703, 1987.—Recent studies indicate that polymorphonuclear neutrophils (PMNs) infiltrate the intestinal mucosa during ischemia and after reperfusion. To determine whether PMNs mediate the increased microvascular permeability produced by ischemia-reperfusion (I/R) we treated cats with either saline, antineutrophil serum (ANS), or a monoclonal antibody specific for the β-chain of the CD18 complex (MoAb 60.3) that prevents neutrophil adherence and extravasation. Intestinal microvascular permeability to plasma proteins was measured in control preparations (0.08 ± 0.007), in preparations subjected to 1 h of ischemia then reperfusion (I/R, 0.32 ± 0.02), I/R preparations treated with ANS (0.13 ± 0.01), and I/R preparations treated with MoAb (0.12 ± 0.003). Our results indicate that both PMN depletion (to <10% control) and prevention of PMN adherence significantly attenuate the increased microvascular permeability induced by I/R. These findings, coupled to previous results obtained from this model, support the hypothesis that neutrophils, which accumulate in the mucosa in response to xanthine oxidase activation, mediate the oxyradical-dependent injury produced by reperfusion of the ischemic bowel.

neutrophil adherence; microvascular permeability; reactive oxygen metabolites; intestine

There is now a substantial body of evidence which indicates that reactive oxygen metabolites (ROM) mediate the microvascular and parenchymal injury associated with reperfusion of ischemic tissues (12, 15). Two enzymes are frequently invoked to explain ROM production after ischemia-reperfusion (I/R), i.e., xanthine oxidase and neutrophilic NADPH oxidase. A role for xanthine oxidase is supported by reports that inhibition (allopurinol, pterin aldehyde) or inactivation (tungsten-supplemented, molybdenum-deficient diet) of the enzyme provides a degree of protection against I/R injury similar to that observed with oxyradical scavengers (5, 6, 15, 17, 18). These observations, coupled to the high activity of the enzyme in mucosal epithelium, has led us to conclude that xanthine oxidase is a major source of ROM produced following reperfusion of the ischemic bowel.

A role for neutrophils in ROM production after I/R was first proposed by Romson and co-workers (21) in studies of the myocardium. They observed that neutrophil depletion reduced infarct size to a similar extent as pretreatment with oxyradical scavengers. Others (3, 24, 25) have also noted an attenuation of I/R injury with neutrophil depletion; however, this beneficial effect was attributed to an improved blood flow at low perfusion pressures due to limited microvascular plugging by neutrophils. Some investigators (23) have been unable to demonstrate protection against I/R injury with neutrophil depletion. Thus the role of neutrophils as a source of ROM following reperfusion of ischemic tissues remains undefined.

Recent work by Grisham and colleagues (9) indicates that neutrophil infiltration into the ischemic small bowel is initiated by xanthine oxidase-derived ROM. They observed that the I/R-induced neutrophil infiltration is largely prevented by pretreatment with either allopurinol or superoxide dismutase. These findings suggest that either 1) xanthine oxidase-derived ROM are primary mediators of I/R injury, and neutrophil infiltration is a secondary event or 2) that xanthine oxidase-derived ROM cause relatively little tissue injury and primarily act to elicit neutrophil infiltration and activation, which is ultimately responsible for tissue injury.

An important question that arises from the data relating neutrophil infiltration to xanthine oxidase-derived ROM is whether neutrophils are a cause or an effect of I/R injury in the small bowel. The present study was undertaken to address this question. Two approaches were used to test whether neutrophils mediate the I/R-induced increase in intestinal capillary permeability, i.e.,
neutrophil depletion with polyclonal antiserum and prevention of neutrophil adherence with a monoclonal antibody (MoAb 60.3) directed against a specific membrane associated glycoprotein (β-chain of CD18 complex) that modulates neutrophil adherence to endothelium (2). Precautions were taken to minimize the influence of improved blood perfusion following neutrophil depletion or prevention of neutrophil adherence. The results of these studies indicate that adherent neutrophils are the primary mediators of the increased microvascular permeability induced by I/R in the small bowel.

METHODS

Surgical procedure. The experimental preparation used in this study is similar, except for minor modifications to that described in detail in previous studies (6, 17). Briefly, 20 cats previously fasted for 18–24 h were initially anesthetized with 50 mg/kg ketamine-HCl. The right femoral artery and vein were cannulated, and anesthesia was maintained by administration of pentobarbital sodium into the femoral vein. Systemic arterial pressure was measured with a Statham P23A transducer (Statham, Oxnard, CA) that was connected to a carotid artery cannula. A tracheotomy was performed to facilitate breathing and as a means of artificial ventilation if the cats failed to breathe spontaneously during the experiment.

A midline abdominal incision was made and a 15- to 20-cm segment of ileum was isolated; blood and lymph vessels were maintained intact. The remainder of the small and large intestine was extirpated. Immediately after isolation of the ileal segment and before cannulation of the lymphatic and blood vessels, heparin (1,000 U/kg) was administered intravenously. Body and gut temperatures were maintained at 37°C with thermistor-controlled infrared lamps. To minimize evaporation and tissue dehydration, the intestinal segment was moistened with saline-soaked gauze and placed in a plastic bag.

A large prenodal lymphatic vessel draining the intestinal segment was cannulated, and lymph flow was determined by observing the movement of lymph in a calibrated micropipette (50–1,000 μl). Lymph (C_L) and plasma (C_P) total protein concentrations were measured with a calibrated refractometer (American Optical, Buffalo, NY). A large cannula was inserted into the superior mesenteric vein, and venous outflow drained into a reservoir mounted on a vertically positioned pulley system. Blood from the reservoir was returned to the animal via the femoral vein cannula. Venous outflow pressure of the intestinal segment was set by adjusting the height of the reservoir and was monitored from a T-connector in the venous circuit. Control venous pressure was set at 0 mmHg. Intestinal blood flow was periodically measured by timed collection of venous effluent in a graduated cylinder.

An arterial circuit was established between the superior mesenteric and femoral arteries. Superior mesenteric arterial pressure was measured via a T-tube interposed within the arterial circuit using a Statham P23A transducer. All pressure cannulas and associated transducers were positioned at heart level. Heparinized whole blood from a donor animal was used to prime all the extracorporeal blood circuits. Systemic and superior mesenteric arterial pressures and venous pressure were continually recorded with a Grass physiological recorder (Grass Instrument, Quincy, MA). At the end of each experiment the intestinal segment was weighed. Lymph flow and blood flow were normalized to milliliters per minute per 100 gram tissue.

Experimental protocols. In all experiments, control values for intestinal blood flow, lymph flow, and C_L and C_P were obtained at a normal local arterial pressure (90–120 mmHg) and at a venous pressure of 0 mmHg. The animals then received either 1.0 ml neutrophil antiserum (n = 6), 2 mg/kg of the murine MoAb 60.3 (n = 6), nonimmune serum (n = 4), or saline (n = 4) injected directly into the superior mesenteric arterial loop. Since the saline and nonimmune serum-treated groups were not significantly different, the data were pooled. Aliquots of whole blood (50 μl) were obtained during the control period and every hour thereafter. The blood samples were diluted with 3% acetic acid to lyse erythrocytes. Nuclei of the leukocytes were stained with crystal violet (0.01%) and polymorphonuclear (neutrophils), and mononuclear (lymphocytes, monocytes) leukocytes were counted using a hemocytometer. Neutrophil counts were expressed as cells per milliliter of whole blood.

One hour after the administration of neutrophil antiserum, MoAb 60.3, nonimmune serum, or saline, local arterial pressure was lowered using an adjustable clamp to partially occlude the local arterial circuit, such that intestinal blood flow was reduced to 15–20% of the control value. Blood flow was maintained at this level for 1 h. After 60 min of ischemia, the intestine was reperfused by releasing the partial arterial occlusion. Once all measured parameters reached a new steady state, venous pressure was elevated in 10-mmHg increments up to 40 mmHg. Intestinal venous pressure was maintained constant at each pressure level until all parameters (lymph flow, C_L, C_P) were in a steady state. This procedure was used in all I/R experiments. The same protocol was used for control estimates of microvascular permeability with the exception that the intestine was not subjected to a period of ischemia.

Microvascular permeability estimates. The osmotic reflection coefficient (σ_d) of intestinal capillaries was estimated using the steady-state relationship between the lymph-to-plasma protein concentration ratio (C_L/C_P) and lymph flow (6, 17). As lymph flow is increased, C_L/C_P rapidly decreases (filtration rate dependent) and then becomes relatively constant at a minimal value (filtration rate independent) when lymph flow is high. At low venous pressures, the exchange of macromolecules across the intestinal capillary wall occurs by both diffusion and convection. Elevation of venous pressure increases the convective movement of macromolecules across the capillary wall while at the same time the diffusive contribution to total exchange is reduced to a negligible level. Theoretical and experimental evidence suggests that σ_d = 1–C_L/C_P when C_L/C_P is filtration rate independent, i.e., when diffusive exchange is negligible (7, 26). In the present study, microvascular permeability was expressed
Preparation of antiserum to feline polymorphonuclear leukocytes (neutrophils). Pooled antiserum to feline neutrophils was raised in four New Zealand White rabbits. Briefly, neutrophils and mononuclear leukocytes were purified by the method of Grisham et al. (8) in which erythrocytes were sedimented with dextran, and mononuclear and polymorphonuclear leukocytes were separated from each other on a Histopaque 1077 gradient. Contaminating erythrocytes were removed by hypotonic lysis. Neutrophils prepared by this method were >90% pure with the other 10% composed of monocytes and an occasional eosinophil. Eight milliliters of washed neutrophils (2 x 10^7 cells/ml) was mixed with 8 ml of Freund’s complete adjuvant for subcutaneous immunization. Each rabbit received a booster injection of neutrophils (4 x 10^7) in Freund’s incomplete adjuvant 21 days after the initial injection. Ten days later, serum was collected, heat inactivated (56°C for 30 min), and exhaustively absorbed against feline pooled erythrocytes and the mononuclear leukocyte fraction. Immunoglobulins were precipitated by (NH₄)₂SO₄ fractionation (40%), and dialyzed against 4 l of phosphate-buffered saline for 48 h at 4°C. The antibody agglutination titer against purified feline neutrophils was assessed using serial dilutions of the immunoglobulin G (IgG) fraction. The highest dilution that showed agglutination of five or more cells was 1:1,024. This IgG antineutrophil fraction was stored at -20°C for subsequent use. Nonimmune serum was generated from plasma obtained from nonimmunized rabbits, which was treated in the above manner.

Neutrophil adherence. Murine MoAb 60.3 to the neutrophil membrane glycoprotein complex CD18 was tested for its effect on adherence of feline neutrophils using the method of Fehr and Dahinden (4). Briefly, 1-ml aliquots of neutrophils (2 x 10⁶ cells/ml in heat-inactivated plasma) were placed into wells of plastic tissue culture plates containing varying concentrations of MoAb 60.3. Plates were incubated for 40 min at 37°C. Each well was then gently rinsed three times with one volume of ice-cold phosphate-buffered saline. Neutrophil adherence was assessed by measuring the myeloperoxidase activity (MPO) of the neutrophil suspensions added to wells and the MPO of neutrophils adhering to the plastic wells after washing. Neutrophil adherence was expressed as percent adherent cells.

RESULTS

Figure 1 illustrates the effects of intra-arterial infusion of antineutrophil serum (ANS), monoclonal antibodies (MoAb 60.3), and nonimmune serum on blood neutrophil count in our experiments. The control mean white blood cell count for all experiments was 1.59 ± 0.13 x 10⁷ cells/ml of blood. In ANS-treated cats, neutrophil depletion was maximal (less 5% of control) 1 h after treatment and remained below 10% of control for the next 3 h. Blood neutrophil count actually increased in the MoAb and nonimmune serum-treated groups.

Figure 2 illustrates the influence of MoAb 60.3 on the adherence of cat neutrophils in vitro. MoAb 60.3 caused a dose-related reduction in neutrophil adherence. The maximal response (80% reduction) to MoAb 60.3 was observed at concentrations ≥5 µg/ml. It is estimated that the dose of MoAb used in the in vivo studies (2 mg/kg) roughly corresponds with the 50 µg/ml concentration in Fig. 2.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Microvascular Permeability (1 - cₒ)</th>
<th>Relative Significance</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.08 ± 0.007</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>I/R-saline or nonimmune serum</td>
<td>0.32 ± 0.02</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>I/R-ANS</td>
<td>0.13 ± 0.01</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>I/R-MoAb 60.3</td>
<td>0.12 ± 0.03</td>
<td>P &lt; 0.001</td>
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I/R, ischemia-reperfusion; ANS, antineutrophil serum; MoAb, monoclonal antibody; cₒ, osmotic reflection coefficient. * Means ± SE.
Table 1 summarizes the microvascular permeability data obtained in the various experimental groups. The results indicate that neutrophil depletion with ANS or prevention of neutrophil adherence with MoAb 60.3 significantly attenuate the increased microvascular permeability induced by I/R.

DISCUSSION

Reactive oxygen metabolites are now considered to be major mediators of the microvascular injury associated with reperfusion of the ischemic intestine (15). The results of several studies indicate that xanthine oxidase, an enzyme that is found both in intestinal epithelia (14) and vascular endothelium (20), is largely responsible for the production of superoxide and hydrogen peroxide at reperfusion (5, 17, 19). Superoxide and hydrogen peroxide react in the presence of catalytically active iron to produce the highly reactive hydroxyl radical, which appears to mediate I/R-induced microvascular injury (13, 17).

Another potential source of ROM following reperfusion of ischemic tissues is the neutrophil. Activated neutrophils produce superoxide by the membrane-associated enzyme, NADPH oxidase (22). Neutrophils are also known to accumulate in the microvasculature during ischemia and after reperfusion (3, 9, 16, 24). It has recently been shown that treatment with either allopurinol or superoxide dismutase largely prevents I/R-induced neutrophil infiltration in the intestine (9). This observation provides the foundation of the hypothesis that xanthine oxidase-derived ROM play an important role in eliciting I/R-induced neutrophil infiltration.

The objective of this study was to determine whether neutrophil infiltration is a cause, rather than an effect, of I/R-induced microvascular injury in the intestine. Two approaches were used to assess the importance of neutrophils in mediating the increased intestinal microvascular permeability associated with I/R, i.e., neutrophil depletion and prevention of neutrophil adherence. The results obtained using both approaches indicate that neutrophils are the primary mediators of I/R-induced microvascular injury. Although this study is the first to clearly demonstrate a role for neutrophils in I/R-induced microvascular injury, others (21, 24, 25) have shown protection against I/R-induced parenchymal cell injury following neutrophil depletion. Interpretation of the results of these studies is complicated by the fact that neutrophil depletion leads to an improved microvascular perfusion during the ischemic insult. This observation is consistent with the notion that granulocyte plugging of capillaries significantly increases microvascular resistance during ischemia (24). The importance of this improvement of microvascular perfusion by neutrophil depletion is best exemplified by the observation that the protection against hemorrhagic shock-induced gastric mucosal injury afforded by antineutrophil serum can be attributed entirely to improved perfusion during the ischemic period (25). Inasmuch as the experimental model used in the present study allows for precise control of total intestinal blood flow, we designed our experiments so as to prevent the improved perfusion during ischemia that results from neutrophil depletion. Our results demonstrate that neutrophil depletion with antineutrophil serum affords significant protection against I/R-induced microvascular injury even in the absence of beneficial hemodynamic changes. Thus our findings, coupled to work by others, suggest that invading neutrophils mediate cell injury during I/R by two mechanisms: 1) by exacerbation of the ischemic insult by plugging the microvasculature, and 2) by releasing cytotoxic substances at or near endothelial and parenchymal cells.

It is now recognized that neutrophil adherence to microvascular endothelium is a prerequisite for extravascular migration at sites of inflammation and may be a critical factor in the pathogenesis of vascular injury associated with neutrophil infiltration (10). There is a growing body of evidence that indicates that a membrane-associated glycoprotein complex (designated CD18) is largely responsible for the leukocyte-dependent augmentation of the adhesive interaction between neutrophils and microvascular endothelium (10). This membrane glycoprotein complex is recognized by the murine MoAb 60.3 (2). Addition of MoAb 60.3 to normal neutrophils induces defects in neutrophil adherence to and chemotaxis across cultured endothelial monolayers (11). Recent in vivo studies demonstrate that MoAb 60.3 prevents neutrophil accumulation and neutrophil-dependent albumin extravasation induced by intradermal injection of chemotactic factors (FMLP, C5a) (1). The same investigators used intravital microscopic techniques to show that MoAb prevented neutrophil sticking in venules induced by surface application of leukotriene B4 (LTB4) and zymosan-activated plasma. We have demonstrated that MoAb 60.3 dramatically reduces the adherence of cat neutrophils to plastic surfaces (see Fig. 2). Given the importance of leukocyte-endothelial interactions in conditions associated with neutrophil infiltration, we examined whether neutrophil adherence to endothelium also plays an important role in the microvascular injury induced by I/R. Our results (Table 1) indicate that prevention of neutrophil adherence with MoAb 60.3 affords significant protection against I/R-induced microvascular injury. The observation that neutrophil depletion and prevention of neutrophil adherence are equally effective in attenuating the microvascular injury suggests that neutrophil adherence is the rate-limiting step in neutrophil-mediated microvascular injury.

Although our results provide strong support for a role of neutrophils in I/R-induced microvascular injury, the chemical mediators of this injury process remain undefined. It is tempting to attribute the injury process entirely to ROM, since oxyradical scavengers also protect against I/R injury (15). However, the latter observation does not constitute strong support for oxyradicals as final mediators of injury, since superoxide dismutase also prevents neutrophil infiltration, indicating that oxyradicals may function primarily to elicit neutrophil infiltration. Activated neutrophils also liberate a number of nonoxidative toxins (e.g., proteases, cationic proteins, collagenase), which are capable of damaging one or more components of exchange vessels. The role of nonoxidative mechanisms in the I/R-induced microvascular injury...
has not been studied yet it clearly warrants attention.

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REFERENCES


