Neutrophil-Mediated Microvascular Dysfunction in Postischemic Canine Skeletal Muscle
Role of Granulocyte Adherence
Donna L. Carden, J. Keith Smith, and Ronald J. Korthuis

Recent studies implicate a role for granulocytes in the genesis of the microvascular and parenchymal cell dysfunction, which occurs upon reperfusion of ischemic tissues. Although the molecular mechanisms underlying this neutrophil-mediated injury are not completely understood, it is clear that an essential first step in granulocyte migration from the vascular lumen to the interstitial space is adherence to vascular endothelium. The purpose of this study was to determine whether prevention of neutrophil adherence with monoclonal antibody IB4, directed against the neutrophil CD11/CD18 glycoprotein adherence complex or neutrophil depletion with a specific polyclonal antineutrophil serum would attenuate the microvascular dysfunction seen in postischemic skeletal muscle. Changes in vascular permeability were assessed by measurement of the solvent drag reflection coefficient for total plasma proteins (\(\sigma\)) in isolated canine gracilis muscle subjected to ischemia/reperfusion, ischemia/reperfusion plus antineutrophil serum, or ischemia/reperfusion plus IB4. Estimates of \(\sigma\) averaged 0.83±0.04 in nonischemic, control gracilis muscles, while ischemia/reperfusion was associated with a marked increase in vascular permeability (decrease in \(\sigma\) to 0.54±0.04) and vascular resistance (increased by 135±41% over the control value). Prevention of neutrophil adherence or neutrophil depletion prevented this increase in vascular permeability (\(\sigma=0.80±0.03\) and 1.01±0.06, respectively) and resistance (decrease of 16.51±8.0% and increase of 2.4±4.6% over control values, respectively). The results of this study suggest that neutrophils play a critical role in the genesis of microvascular dysfunction in postischemic skeletal muscle. Furthermore, neutrophil adherence to vascular endothelium appears to be a prerequisite for the production of this injury. (Circulation Research 1990;66:1436–1444)

When skeletal muscle is subjected to a prolonged period of ischemia followed by reperfusion, several functional and morphological changes occur including impaired ability to develop tension, mitochondrial swelling, disruption of sarcoplasmic organization, leakage of cytosolic enzymes into the circulation, endothelial cell swelling and denudation, and the development of the no-reflow phenomenon.\(^1\)–\(^10\) These alterations are not solely due to the interruption of blood flow or to the accumulation of potentially toxic metabolic end products during the ischemic period.\(^4\)–\(^6\),\(^11\) Rather, it appears that reperfusion initiates a complex sequence of events, including the generation of reactive oxygen metabolites, liberation of proinflammatory mediators, and granulocyte adherence, infiltration, and activation, which leads to the production of microvascular and parenchymal cell injury in postischemic skeletal muscle.\(^1\)–\(^5\),\(^12\)

It has been suggested that polymorphonuclear leukocytes represent an important potential source of reactive oxygen metabolites and cellular dysfunction in postischemic tissues.\(^3\),\(^13\)–\(^20\) This notion is based in part on the observation that activated granulocytes produce reactive oxygen metabolites via the NADPH oxidase system and also secrete myeloperoxidase, an enzyme that catalyzes the production of the potent cytotoxic oxidants, hypochlorous acid, and

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Moreover, activated neutrophils are capable of damaging endothelial cells, and they increase endothelial cell monolayer permeability through the production of cytotoxic oxidants and nonoxidative toxins (e.g., proteases, cationic proteins, and collagenase). Recently, it has been demonstrated that reperfusion of ischemic skeletal muscle is associated with a dramatic increase in skeletal muscle neutrophil content after 4 hours of ischemia and 1 hour of reperfusion. Thus, activated granulocytes are not only capable of generating cytotoxic oxidants and producing microvascular injury, but they also accumulate in large numbers in postischemic skeletal muscle. These results provide indirect support for the notion that neutrophils are involved in the microvascular dysfunction that occurs when skeletal muscle is reperfused after prolonged ischemia.

More direct support for a role for granulocytes in postischemic skeletal muscle injury is provided by our observation that leukocyte depletion (primarily neutrophils) using Leukopak filters (Baxter-Travenol, Deerfield, Illinois) attenuated reperfusion-induced increases in microvascular permeability and muscle vascular resistance. This finding was subsequently confirmed by Klausner et al, who demonstrated that reperfusion-induced increases in skeletal muscle lymph flow and protein concentration are largely attenuated in animals rendered neutropenic by treatment with hydroxyurea or nitrogen mustard. More recently, Belkin et al have demonstrated a significant reduction in the extent of postischemic muscle necrosis in animals in which the circulating leukocyte count was reduced by x-irradiation.

Although the aforementioned studies support the concept that neutrophils play a major role in postischemic injury in skeletal muscle, the molecular events involved in the production of neutrophil-mediated injury are not well understood. However, it is well established that granulocyte adherence to microvascular endothelium is an essential first step in the emigration of neutrophils from the vascular to extravascular compartment. This adhesive interaction between activated granulocytes and microvascular endothelium depends in part on the expression of a neutrophil membrane-associated glycoprotein adherence complex (the CD11/CD18 adherence complex). Neutrophil adherence may be a critical factor in the development of microvascular damage associated with neutrophil infiltration in postischemic skeletal muscle. For example, the intimate contact between activated neutrophils and endothelial cells that results during neutrophil adhesion may be necessary for the production of endothelial cell injury by neutrophils in response to proinflammatory stimuli. In addition, ischemia/reperfusion-induced neutrophil adherence to microvascular endothelium has been implicated as a causative factor in the development of the no-reflow phenomenon.

The development of monoclonal antibodies that recognize epitopes on the CD11/CD18 glycoprotein adherence complex and inhibit the adhesive interaction between activated granulocytes and microvascular endothelium represents an attractive and highly specific means to probe the role of neutrophil adherence in the genesis of granulocyte-dependent postischemic microvascular injury. Thus, the purpose of this study was to determine whether prevention of neutrophil adherence with monoclonal antibody IB, directed against the β-chain of the CD11/CD18 glycoprotein adherence complex would attenuate the microvascular dysfunction associated with reperfusion of ischemic skeletal muscle. In a further attempt to more precisely define the role of neutrophils in the microvascular dysfunction produced by ischemia/reperfusion in skeletal muscle, we also evaluated the effects of selective depletion of circulating neutrophils by use of a highly specific polyclonal antineutrophil serum (ANS).

Materials and Methods

Surgical Preparation

Adult mongrel dogs of either sex were anesthetized with intravenous pentobarbital sodium (30 mg/kg), intubated, and ventilated with room air. The left external jugular vein and carotid artery were cannulated for fluid or drug administration and for arterial pressure monitoring, respectively. The gracilis muscle was surgically prepared as described previously. In brief, the skin overlying the right gracilis muscle was divided, and the gracilis muscle was freed from surrounding connective tissue by blunt dissection. The minor vascular pedicle near the muscle insertion was ligated, and any collateral vessels supplying the muscle via the origin and insertion were occluded with tight ligatures. The obturator nerve was sectioned. After ligating all collateral vessels arising from the proximal caudal femoral artery and vein, the muscle was allowed to stabilize for 30 minutes. Heparin (10,000 units) was then administered, and the proximal caudal femoral artery was cannulated with PE-190 tubing and connected to an artificial perfusion system driven by a pump (Minipuls 2, Gilson Medical Electronics, Middleton, Wisconsin) that maintained constant flow through the gracilis muscle. The proximal caudal femoral vein was cannulated with PE-205 tubing with the outflow directed to a reperfusion reservoir. Arterial and venous pressures were continuously measured from a side branch of the arterial and venous pressure catheters, respectively, using strain-gauge transducers. Blood flow was adjusted to maintain muscle perfusion pressure at 100 mm Hg. Blood flow was determined by timed collection of venous outflow in a graduated cylinder. Vascular isolation was assumed complete if arterial perfusion pressure decreased to less than 20 mm Hg when the perfusion pump was turned off. The gracilis muscle was perfused with autologous heparinized blood, which was gassed with a mixture of 95% O2-5% CO2. Perfusate and muscle temperature were measured using a dual-reading telethermometer.
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Blood and protein concentration, respectively. The hematocrit and plasma protein concentration of the perfusing blood were determined before and after a period of filtration induced by elevation of venous pressure. \( \sigma \) may then be estimated using the equation:

\[
\sigma = 1 - \left\{ \frac{(C_0/C_f) \left[ 1 - \left(1 - (H_1/H_2)\right) \frac{C_i(C_f - C_i)}{C_f - C_i} \right] + (V_i/V_f) \left( 1 - (H_1/H_2) \right) \frac{C_i(C_f - C_i)}{C_f - C_i} \right\}
\]

where \( H_1 \) and \( H_2 \) are the initial and final hematocrits, respectively, and \( C_0, C_f, \) and \( C_i \) are the initial, final, and average plasma protein concentrations, respectively. Hematocrits were determined by the microhematocrit method, and plasma total protein was determined by refractometry (Reichert Scientific Instruments, Buffalo, New York). Four blood samples (25 \( \mu \)l each) were obtained for each hematocrit and protein concentration, and the averages of these four measurements were used for the estimation of \( \sigma \). If hemolysis, which decreases red blood cell volume and increases plasma hemoglobin (and thus increases plasma total protein concentration), occurs during the period of increased filtration, a systematic overestimation of \( \sigma \) may occur.

Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was determined by a modification of the method of Grisham et al.\textsuperscript{29} Muscle samples were homogenized in 20 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA using a polytron tissue homogenizer. Two milliliters of muscle homogenate was centrifuged at 20,000g for 15 minutes at 4°C to pellet the insoluble cellular debris. The supernatant, which contains less than 5% of the total MPO activity and greater than 95% of water-soluble hemeproteins (myoglobin and hemoglobin), was discarded. The pellet was then rehomogenized in an equivalent volume of 0.05 M potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HETAB). The samples were freeze-thawed two times. MPO activity was assessed by measuring the \( H_2O_2 \)-dependent oxidation of tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO present that causes a change in absorbance of 1.0/min at 655 nm and 37°C.

Neutrophil Adherence

The monoclonal antibody IB\textsubscript{4}, directed against the neutrophil membrane CD18 glycoprotein adherence
complex, was provided as a generous gift by Dr. Karl Aflors of Pharmacia Laboratories, La Jolla, California. IB₄ was tested for its effect on adherence of canine neutrophils using a modified method of Fehr and Dahinden.³⁰ Briefly, 500-µl aliquots of ⁵¹Cr-labeled neutrophils (2×10⁶ cells/ml) in heat-inactivated plasma were placed into plastic tissue culture plates containing varying concentrations of IB₄ (0–20 µg/ml). The wells were incubated for 40 minutes at 37°C with 4×10⁻⁷ M phorbol myristate acetate. Each well was gently washed three times with ice-cold phosphate-buffered saline. The cells in each well were then lysed by incubation for 15 minutes with 500 µl of 1% HETAB. Neutrophil adherence was assessed by measuring ⁵¹Cr activity in the cell lysate using the following formula:

\[
\text{% adherence} = \frac{\text{lysate (cpm)} \times 100}{\text{whole neutrophils (1×10⁶ cells) (cpm)}}
\]

**Experimental Protocols**

**Group 1: Ischemia/reperfusion.** After the surgical preparation described above was completed, the muscles were allowed to stabilize for 15–30 minutes. Then, initial arterial and venous pressures and blood flow measurements were recorded, and vascular resistance was calculated. Arterial inflow was occluded for 4 hours (n=5) while the muscle preparation was maintained between 36° and 38°C (normothermic ischemia). At the conclusion of the ischemic period, blood flow was re instituted and adjusted to maintain muscle perfusion at 100 mm Hg. Thirty minutes after reinstitution of blood flow, arterial and venous pressures and blood flow were recorded; vascular resistance was calculated. After obtaining these data, α was estimated.

**Group 2: Nonischemic control.** Animals in this group (n=5) were treated in an identical fashion to those in group 1 except that gracilis muscle perfusion was maintained for the duration of the experiment (no ischemia was produced).

**Group 3: Ischemia/reperfusion plus neutropenia.** Before isolation of the gracilis muscle, animals (n=5) were rendered neutropenic with a continuous (3 ml/hr) intravenous infusion of canine specific antineutrophil serum. Aliquots of whole blood (50 µl) were obtained for peripheral neutrophil counts before and at 30-minute intervals after initiating ANS infusion. The blood samples were diluted with 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 hemocytometer, and neutrophil counts were expressed as cells per milliliter of whole blood. The surgical preparation was begun when the peripheral leukocyte count was reduced to less than 5% of the control value. The remainder of the surgical procedures and experimental protocols were conducted in an identical fashion as those described for group 1 above.

**Group 4: Ischemia/reperfusion plus IB₄.** These studies were completed as described for group 1 except that monoclonal antibody IB₄ (40 µg/ml) was administered to the perfusion reservoir just before the onset of reperfusion.

**Statistical Analysis**

Data were analyzed by comparison of means by using a one-way analysis of variance. Scheffe’s test was performed for evaluation of significant differences. Significance was defined as p<0.05, and paired analyses were used where appropriate.

**Results**

**Hematologic Data After Neutrophil Depletion**

Figure 1 illustrates the effect of continuous intravenous infusion of ANS on peripheral neutrophil count. Neutrophil depletion with ANS was maximal (less than 5% of control) at 45 minutes after initiation of infusion and was sustained at this level for the remainder of the experimental protocol. Table 1 illustrates that ANS was without effect on the other measured hematologic parameters.

**Neutrophil Adherence Data**

Figure 2 illustrates the influence of monoclonal antibody IB₄ on the adherence of dog neutrophils in vitro. IB₄ caused a dose-related reduction of neutrophil adherence to plastic. The maximal response,

![Figure 1](http://circres.ahajournals.org/content/8/8/1439/F1.large.jpg)

**Figure 1.** Graph showing effect of continuous intravenous infusion of antineutrophil serum (ANS) on peripheral blood neutrophil counts.

**Table 1.** Hematologic Parameters Obtained Before and After Neutrophil Depletion With Canine-Specific Antineutrophil Serum and After Administration of Monoclonal Antibody IB₄

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ANS</th>
<th>IB₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (%)</td>
<td>37.5±2.3</td>
<td>39.7±3.9</td>
<td>35.8±1.5</td>
</tr>
<tr>
<td>WBC/mm³ (×10⁶)</td>
<td>6.3±0.7</td>
<td>0.4±0.2*</td>
<td>5.7±0.5</td>
</tr>
<tr>
<td>Platelets/mm³ (×10³)</td>
<td>235±35</td>
<td>175±17</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. WBC, white blood cell; Control, values obtained before neutrophil depletion; ANS, values obtained after neutrophil depletion with canine-specific antineutrophil serum; IB₄, values obtained after administration of the monoclonal antibody specific for the CD11/CD18 adherence complex on neutrophils; ND, not determined.

*p<0.05 relative to control.
greater than 99% reduction of adherence, was observed at IB₄ concentrations of 10 μg/ml or more.

**Tissue Myeloperoxidase Activity**

Figure 3 depicts the tissue myeloperoxidase activity obtained in gracilis muscles subjected to ischemia/reperfusion, ischemia/reperfusion plus IB₄, and ischemia/reperfusion plus ANS. Tissue MPO activity increased significantly in muscles subjected to 4 hours of ischemia and 1 hour of reperfusion (8.8±1.7 units/g wet wt) when compared with muscles treated with IB₄ or ANS (2.8±1.0 units/g wet wt and 2.3±0.5 units/g wet wt, respectively). We have previously shown that tissue MPO activity is not significantly increased over control values in muscles subjected to 4 hours of ischemia alone.¹² These results suggest that skeletal muscle reperfusion is associated with a dramatic influx of tissue neutrophils. This postischemic neutrophil infiltration is effectively prevented by either neutrophil depletion or prevention of neutrophil adherence.

**Vascular Permeability Data**

Figure 4 summarizes σ data obtained in the various groups. In nonischemic, continuously perfused gracilis muscles (n=5), σ averaged 0.83±0.04. σ was reduced to 0.54±0.04 in preparations (n=5) subjected to ischemia/reperfusion and phosphate-buffered saline vehicle alone. Estimates of σ obtained from animals rendered neutropenic with ANS (n=5) or treated with IB₄ before reperfusion (n=5) were not significantly different from nonischemic controls (1.01±0.06 and 0.80±0.03, respectively).

**Vascular Resistance Data**

Muscle vascular resistances measured before induction of ischemia and after 30 minutes of reperfusion in the various groups are presented in Table 2. Although preischemic resistances were fairly variable, the mean resistances all fell within the range we normally obtain, and the mean values for the four groups were not different when determined by analysis of variance. Although muscle vascular resistance was not altered by 4.5 hours of continuous perfusion (equivalent to the time period of ischemia/reperfusion), ischemia/reperfusion caused an increase in vascular resistance of 135±41% when compared with preischemic values. This increase in resistance was prevented by neutropenia or the presence of IB₄ in the perfusion reservoir at the time of reperfusion. These results suggest that the rise in resistance associated with the reperfusion
of ischemic gracilis muscles (a manifestation of the no-reflow phenomenon) is neutrophil dependent and may be mediated by adherence of granulocytes to the microvascular endothelium.

**Discussion**

A role for granulocytes in the genesis of ischemia/reperfusion injury was first proposed by Romson et al.\(^\text{14}\) These investigators noted that neutrophil depletion with antineutrophil serum was as effective as administration of oxidant scavengers in reducing infarct size in postischemic myocardium, a result that suggests that granulocytes produce reperfusion injury through the production of reactive oxygen metabolites. Recent studies conducted both in the heart and other tissues have confirmed a role for granulocytes in postischemic tissue injury.\(^\text{13,15,31}\) However, the beneficial effect noted in these latter studies was attributed to an improvement in blood flow at low arterial pressures secondary to an attenuation of leukocyte capillary plugging. Subsequent work by Hernandez et al.\(^\text{32}\) indicates that neutrophil depletion provides significant protection against reperfusion injury even in the absence of an improvement in blood flow. Based on these observations, it seems clear that neutrophils contribute to postischemic tissue injury by at least two mechanisms: 1) extension of the ischemic insult by leukocyte capillary plugging and 2) release of cytotoxic substances (e.g., reactive oxygen metabolites, proteases, and collagenase) near cells.

Recently, we\(^\text{3,12}\) and others\(^\text{16,17}\) have provided evidence that extends these observations to skeletal muscle. For example, reperfusion of ischemic canine gracilis muscles is associated with a rapid and marked increase in muscle neutrophil content.\(^\text{12}\) More direct support for the notion that neutrophils play a role in the genesis of postischemic microvascular dysfunction is provided by our observation that leukocyte depletion (primarily neutrophils) using Leukopak filters attenuated reperfusion-induced increases in microvascular permeability and muscle vascular resistance.\(^\text{3}\) This result was subsequently confirmed by Klausner et al.\(^\text{16}\) who demonstrated that white blood cell depletion by administration of the antitumor agents hydroxyurea or nitrogen mustard ameliorated the postischemic increase in skeletal muscle lymph flow and lymph protein concentration. Finally, the extent of muscle necrosis is significantly reduced in animals rendered neutropenic by x-irradiation.\(^\text{17}\)

Although the aforementioned results support the concept that granulocytes play a role in the pathogenesis of postischemic reperfusion injury in skeletal muscle, these studies suffer from the relative non-specificity of the interventions used to produce neutrophil depletion. For example, use of Leukopak filters to deplete neutrophils also effectively depletes circulating platelets.\(^\text{13}\) Indeed, when we became aware of this finding, we conducted experiments to determine the extent of platelet depletion when blood was filtered through the Leukopak filters in our preparation and found that the circulating platelet count was reduced to less than 10% of control (unpublished observations). Although a potential role for platelets seems unlikely since platelets do not accumulate in postischemic skeletal muscle\(^\text{33}\) and are not activated to produce thromboxane during ischemia,\(^\text{34}\) platelets have been implicated in the development of the no-reflow phenomenon,\(^\text{8}\) and platelet-derived substances can increase microvascular permeability.\(^\text{35}\) Thus, the results obtained in studies in which Leukopak filters are used to produce neutropenia must be interpreted with caution.

Similarly, since hydroxyurea, nitrogen mustard, and x-irradiation reduce all types of leukocytes (granulocytes, lymphocytes, and monocytes), it is difficult to determine which cell line participates in the genesis of ischemia/reperfusion injury. Moreover, the generalized effects associated with intravenous administration of the antitumor agents and whole-body irradiation raise the possibility that some as yet unrecognized effect of these perturbations contributed to the attenuation of injury. Thus, we felt it was imperative to precisely define the role of neutrophils in postischemic skeletal muscle injury. To achieve this aim, we administered a highly specific canine ANS before the ischemic insult. Pretreatment with ANS effectively depleted circulating neutrophils, did not significantly reduce platelet counts, and markedly attenuated the increase in vascular permeability and resistance seen in muscles reperfused with whole blood. This result suggests that granulocytes play an active role in the genesis of this microvascular dysfunction and indicates the granulocyte infiltration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Preischemia (mm Hg/ml/min/100 g)</th>
<th>Postischemia (mm Hg/ml/min/100 g)</th>
<th>Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonischemic</td>
<td>18.1±5.1</td>
<td>17.7±4.8</td>
<td>1.9±6.4</td>
</tr>
<tr>
<td>I/R</td>
<td>11.4±1.4</td>
<td>28.1±12.1*</td>
<td>134.8±40.8*</td>
</tr>
<tr>
<td>I/R+ANS</td>
<td>21.3±2.0</td>
<td>22.0±2.6</td>
<td>2.3±4.6</td>
</tr>
<tr>
<td>I/R+IB(_k)</td>
<td>15.2±2.5</td>
<td>13.0±2.9</td>
<td>−16.7±8.0</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Nonischemic, muscles under continuous perfusion for 4.5 hours; I/R, muscles subjected to ischemia/reperfusion alone; I/R+ANS, muscles subjected to ischemia/reperfusion plus antineutrophil serum; I/R+IB\(_k\) muscles subjected to ischemia/reperfusion plus antiadherence monoclonal antibody IB\(_k\). *Statistically different from values obtained before ischemia.
into postischemic skeletal muscle is a cause, rather than an effect, of ischemia/reperfusion injury in this tissue.

Adherence of neutrophils to vascular endothelium is an essential step in the process of granulocyte emigration into the interstitial space. It is well established that chemotactic peptides (C5a) and lipid mediators (leukotriene B4 and platelet-activating factor) increase the adherence of neutrophils to vascular endothelium through a process that is dependent on the expression of a family of neutrophil membrane glycoproteins (MAC-1, LFA-1, P, 150,95) that modulate neutrophil adheriveness. These glycoproteins are α,β, heterodimers that possess distinct α-subunits but share a common β-subunit and are collectively referred to as the CD11/CD18 complex. Inasmuch as ischemia/reperfusion injury is becoming increasingly recognized as a form of acute inflammation in which both chemotactic peptides and lipid mediators are released, it is important to determine whether granulocyte adherence plays an important role in the genesis of this injury. To address this issue, we administered the monoclonal antibody IB4 to the perfusion reservoir at the onset of reperfusion. IB4 is a murine immunoglobulin G, antibody that recognizes an epitope on the β-chain of the neutrophilic membrane glycoprotein adherence complex that is designated CD11/CD18.6 IB4 inhibits activated neutrophils from adhering to plastic (Figure 2) and venular endothelium but does not affect the peripheral neutrophil count (Table 1). In addition, while IB4 and monoclonal antibody 60.3 (another antibody directed against the CD18 complex) have been shown to inhibit stationary adherence, the antibodies do not effect the weaker adhesion associated with leukocyte rolling. Monoclonal antibodies that recognize the CD11/CD18 complex have also been shown to inhibit neutrophil adherence to and chemotaxis across endothelial cell monolayers grown in culture as well as to prevent granulocyte adherence to venular endothelium, neutrophil accumulation in the interstitial space, and neutrophil-dependent albumin leakage induced by administration of proinflammatory agents. Our results indicate that administration of IB4 was remarkably effective in attenuating the increase in microvascular permeability and resistance to blood flow noted in postischemic skeletal muscle. Thus, it appears that adherence of neutrophils to the microvascular endothelium is a prerequisite for granulocyte-mediated reperfusion injury in skeletal muscle. Moreover, the significant decrease in muscle myeloperoxidase activity, a sensitive marker of tissue neutrophil content, noted in IB4-treated gracilis muscles confirms the critical role of neutrophil membrane adherence glycoproteins in the process of granulocyte extravasation.

Neutrophil adherence to microvascular endothelium also appears to be a prerequisite for the production of the vascular injury by granulocytes activated by exposure to proinflammatory stimuli. For example, phorbol myristate acetate–stimulated neutrophils increase the permeability of endothelial cell monolayers and isolated perfused lungs. The increase in permeability noted in cultured endothelial cells was eliminated when the activated neutrophils were separated from the monolayer by a micropore filter. In addition, neutrophil-dependent microvascular injury in isolated perfused lung preparations was attenuated by inhibition of neutrophil adherence with cytochalasin B, 2% dextran, or a monoclonal antibody directed against a neutrophilic membrane glycoprotein that facilitates granulocyte adhesion. These studies suggest that close approximation of the neutrophil to the endothelial cells is required for the production of injury. It also appears that injury to parenchymal cells by activated granulocytes requires close apposition of the activated neutrophil with the target cell. More relevant to the present study are the observations of Simpson et al., who demonstrated that administration of a monoclonal antibody (Mo1 antibody) that binds to the neutrophil CD11b/CD18 complex (α-chain) before reperfusion reduced myocardial infarct size by 46%. Similarly, Hernandez et al. demonstrated that a monoclonal antibody (MoAb 60.3) directed against the glycoprotein adhesion complex of cat neutrophils largely attenuated postischemic intestinal injury. The results of these studies, when coupled with our observations, suggest that neutrophil adherence may be a common feature in the pathogenesis of ischemia/reperfusion injury in a variety of tissues.

The results of our study also have important implications regarding the mechanism underlying the development of the no-reflow phenomenon (i.e., some capillaries fail toperfuse on reinstitution of blood flow) in skeletal muscle. That is, we noted that neutrophil depletion or inhibition of granulocyte adherence ameliorates reperfusion-induced increases in skeletal muscle vascular resistance, a manifestation of no-reflow. We noted a similar attenuation in an earlier study in which neutrophil depletion was accomplished by use of Leukopak filters. These results support the notion that the no-reflow phenomenon may be due, at least in part, to leukocyte capillary plugging. Moreover, the studies in which granulocyte adherence was inhibited by administration of IB4 suggest that granulocyte adherence to the microvascular endothelium, rather than simple physical impaction, may be a prerequisite for the genesis of the no-reflow phenomenon in skeletal muscle.

The neutrophil-dependent increases in microvascular permeability and muscle vascular resistance have important consequences for the pathogenesis of postischemic injury in skeletal muscle. That is, since many muscles are enclosed by a tight fascial sheath, the formation of interstitial edema during reperfusion can be accompanied by a marked rise in interstitial fluid pressure (>60–70 mm Hg), which, in turn, may lead to compressive necrosis, nerve constriction, or rhabdomyolysis. This may necessitate decompressive fasciotomy, excision of necrotic tissue,
or even amputation of the affected limb. Indeed, Labbe et al. have noted that most of the necrosis that develops in canine gracilis muscle subjected to prolonged ischemia followed by reperfusion occurs in the central core of the muscle, and they have attributed this finding to the greater vulnerability of the central region to the compressive effect of edema. Inasmuch as the development of the no-reflow phenomenon may limit the delivery of oxygen and nutrients during reperfusion, local ischemia and hypoxia may persist during reperfusion despite adequate arterial pressures, thereby contributing to further loss of potentially viable muscle fibers. Thus, the microvascular dysfunction produced by ischemia/reperfusion is a significant cause of morbidity in postischemic skeletal muscle. Interventions directed at inhibition of granulocyte adherence may allow for increased salvage of ischemic skeletal muscle by reducing the extent of microvascular dysfunction during reperfusion.

Since the attenuation of postischemic microvascular injury associated with administration of antineutrophil serum and IB₄ was as effective as that which we have been able to achieve in earlier studies with oxidant scavengers, such as superoxide dismutase, or inhibitors of oxidant production, such as allopurinol, it is tempting to attribute the process whereby cells become injured in postischemic muscle to the generation of reactive oxygen metabolites. Indeed, Shasby et al. have presented evidence that activated neutrophils increase the permeability of endothelial cell monolayers and isolated perfused lungs by an oxidant-dependent mechanism. However, this interpretation is complicated by the fact that superoxide dismutase and allopurinol also act to attenuate the increase in tissue neutrophil content in postischemic intestine. A result that suggests that oxidants may function primarily to promote neutrophil infiltration. Activated granulocytes also release a variety of granular enzymes including elastase, collagenase, and cathepsin G, which are capable of degrading the capillary basement membrane and endothelial cell glycocalyx. By disrupting these components of the microvascular barrier, neutrophilic granular enzymes may contribute to reperfusion-induced increases in microvascular permeability. Clearly, evaluation of the potential role of these and other nonoxidative mechanisms in the genesis of ischemia/reperfusion–induced microvascular injury is warranted.

In summary, the results of this study suggest that granulocytes play a critical role in the genesis of ischemia/reperfusion–induced increases in vascular permeability and resistance in skeletal muscle. Moreover, it appears that neutrophil adhesion to vascular endothelium is a prerequisite for the production of this microvascular dysfunction as well as for migration of these phagocytic cells from vascular lumen to the extravascular space in postischemic skeletal muscle.

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