Age-dependent responses of the mesenteric vasculature to ischemia-reperfusion

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Harris, Norman R., and Kimberly W. Langlois. Age-dependent responses of the mesenteric vasculature to ischemia-reperfusion. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H1509–H1515, 1998.—The age-dependent responses of the mesenteric vasculature to ischemia-reperfusion (I/R) were compared in 2-mo-old and 2-yr-old rats. Measurements were made of leukocyte adherence, albumin leakage, and oxidative stress in postcapillary venules. In young rats I/R induced an increase in leukocyte adherence and albumin leakage, but in aged rats I/R induced an increase in albumin leakage without an increase in leukocyte adherence. Furthermore, I/R-induced oxidative stress was higher in the aged rats than in the young rats. To investigate whether the age-associated oxidative stress is related to a decrease in the role of nitric oxide, N\textsuperscript{n}-nitro-L-arginine methyl ester (L-NAME) was superfused onto the mesentery of young and aged rats. L-NAME induced an increase in postcapillary protein leakage only in young rats; however, arteriolar constriction induced by L-NAME occurred in both age groups. These results suggest that different mechanisms contribute to the inflammatory responses and microvascular dysfunction elicited by I/R in young and aged rats.

microvascular permeability; aging; endothelial barrier dysfunction

AGING IS A MAJOR RISK FACTOR for a variety of ischemic disorders including ischemic heart disease and stroke. Intense research over the past decade into ischemia-reperfusion (I/R) injury has implicated a general mechanism whereby oxygen radicals produced at the onset of reperfusion overwhelm endogenous antioxidants, recruit neutrophils to the endothelial wall, and increase vascular permeability, often to the extent of causing inflammation and edema. The central role of neutrophils in this mechanism has been demonstrated by many animal studies in which interference with the adhesive interactions between neutrophils and endothelial cells (rolling and firm adherence) protects the microvasculature from increased permeability. For example, Kurose et al. (14), looking at the mesenteric microcirculation in 2- to 3-mo-old rats, observed 1) a significant increase in reperfusion-induced postcapillary leukocyte adherence and albumin leakage, 2) a high correlation between the number of adherent leukocytes and the extent of albumin leakage, and 3) a significant attenuation of albumin leakage when the rats were injected with antibodies against molecules involved in neutrophil-endothelial cell adhesion.

Much of our knowledge regarding I/R injury comes from animal models, and the animal used most frequently in these studies is the rat. Despite the fact that I/R disproportionately affects aged individuals, young rats are usually chosen in models of I/R injury because of their greater availability, lower cost, and fewer health problems. Results obtained from young animals demonstrate a central role for neutrophils in I/R-induced increases in microvascular permeability; however, it is not clear whether these findings apply to older animals. Several studies have indicated that neutrophils isolated from aged individuals exhibit attenuated chemotaxis (1, 23), oxidant production (7, 20), and phagocytosis (1, 23), and it has been suggested that the attenuation of these functions is caused by an age-associated oxidative stress. Therefore, compared with results seen in young animals subjected to I/R, the neutrophils of aged animals may have a more limited capacity to damage the microvasculature.

Reperfusion of ischemic organs results in the production of toxic reactive oxygen species including superoxide. As a defense mechanism, tissues contain endogenous antioxidants and radical scavengers such as superoxide dismutase and catalase. Many investigators also believe that the presence of endothelial nitric oxide (NO) is protective, in that it efficiently scavenges superoxide (11) and because its production via administration of NO donors or L-arginine (a precursor of NO) is capable of attenuating I/R injury (13, 22). Numerous reports of an age-dependent decrease in endothelium-dependent relaxation (19, 26), a process thought to be mediated by NO, suggest that the role of NO is decreased in aged animals. This hypothesis is further supported by evidence of an age-associated decline in levels of the NO precursors aspartic acid, citrulline, and arginine (27). Therefore, a decline in the function of NO with age may play a prominent role in I/R injury in aged animals.

The primary objective of this study was to characterize and compare reperfusion-induced microvascular events in young and aged rats, including 1) leukocyte adhesion (rolling and firm adherence), 2) albumin leakage, and 3) oxidative stress. Furthermore, the potential role of NO, as a function of age, was studied via NO synthase inhibition.

MATERIALS AND METHODS

Animal preparation. Male Fischer 344 rats ranging in age from 2 to 24 mo were fasted for 18–24 h before surgery. Thiobutabarbital (Inactin; 135 mg/kg body wt) was injected intraperitoneally to induce anesthesia. A tracheotomy was performed on each rat to facilitate breathing throughout the experiment, and the right carotid artery was cannulated for pressure measurements (Pressure Monitor BPI-B, World Precision Instruments, Sarasota, FL) and for blood withdrawal. The left jugular vein was also cannulated for injection of fluorescently labeled albumin and to inject a lethal dose (160 mg/kg) of pentobarbital sodium at the end of the experiment.
Leukocyte adherence was defined as the number of leukocytes that traveled past a selected cross section of venule per minute, \( R_{\text{flux}} \), and the leukocyte rolling velocity, \( V_{\text{RBC}} \) (µm/s): \( R_{\text{flux}} = (R_{\text{flux}}V_{\text{RBC}}) \times (1 \text{ min/60 s} \times 100 \mu m). \) 

A midline abdominal incision was made to allow a section of mesentery from the small intestine to be exteriorized. The rat was placed on its right side on an 8 × 11-in. Plexiglas board, which allowed the selected section of mesentery to be placed over a glass slide mounted on a hole centered in the Plexiglas. The board was mounted onto the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan) equipped with a ×40 objective (Nikon Fluor 40, 0.85 NA) that produced an image captured on videotape (BR-S601 MU videocassette recorder, JVC). The time and date were displayed on the taped image and the live image (Trinicon monitor, Sony) with a time-date generator (WJ-810, Panasonic). A color camera (VK-C150, Hitachi) was used to capture bright-field images, and fluorescence was observed using a combination of an intensifier (model C2400–68, Hamamatsu, Hamamatsu City, Japan) and black and white charge-coupled device camera (model C2400–60, Hamamatsu). Both cameras were simultaneously mounted on the microscope with a dual optical path tube (Nikon).

The mesentery was superfused at 2 ml/min (Minipuls 3 pump, Gilson, Middleton, WI) with bicarbonate-buffered saline bubbled with a 95% N\(_2\)-5% CO\(_2\) gas mixture to reduce the oxygen tension to a physiological level (40-50 mmHg). The superfusing buffer consisted of (in mM) 132 NaCl, 4.7 KCl, 1.2 MgSO\(_4\), 20 NaHCO\(_3\), and 2.0 CaCl\(_2\) and was heated to 37°C before reaching the mesentery by passing through a heat exchanger. Rectal temperature was maintained near 37°C with an infrared heat lamp placed over the rat.

I/R protocol. Before the intestine was exteriorized, a 6- to 8-in. section of vinyl tubing (clear vinyl medical grade; ID 0.5 mm, OD 0.8 mm) was placed around the superior mesenteric artery (SMA) and exteriorized through a 16-gauge needle temporally penetrating the left upper abdominal wall. Both ends of the tubing were fed through a short length (0.5 in.) of polyethylene tubing (PE-40; ID 1.67 mm, OD 2.42 mm), which aided in producing ischemia as follows. The vinyl tube was pulled taut around the SMA and clamped behind the artery (SMA) and exteriorized through a 16-gauge needle (11-in. Plexiglas). The board was mounted onto the stage of an inverted microscope (Nikon Fluor 40, 0.85 NA) that produced an image captured on videotape (BR-S601 MU videocassette recorder, JVC). The time and date were displayed on the taped image and the live image (Trinicon monitor, Sony) with a time-date generator (WJ-810, Panasonic). A color camera (VK-C150, Hitachi) was used to capture bright-field images, and fluorescence was observed using a combination of an intensifier (model C2400–68, Hamamatsu, Hamamatsu City, Japan) and black and white charge-coupled device camera (model C2400–60, Hamamatsu). Both cameras were simultaneously mounted on the microscope with a dual optical path tube (Nikon).

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Measurements from blood samples. Blood samples (400 µl) were taken during the baseline period and after I/R via the carotid arterial cannula, with ~5 µl of 1,000 U/ml heparin added to prevent coagulation. Fifty microliters of the blood were mixed with ten microliters of 1% crystal violet and four hundred forty microliters of 3% acetic acid, and the resulting solution was placed on a hemocytometer to allow the number of leukocytes to be counted. The remainder of the blood sample was spun in a centrifuge, and 50 µl of plasma were drawn off and placed in a refractometer to measure plasma protein concentration. In some experiments, an additional 50 µl of plasma were used for cholesterol measurement (kit from Sigma Chemical, St. Louis, MO).

Observations with bright-field microscopy. Three relatively straight, nonbranched segments of postcapillary venules with lengths of 100 µm and diameters between 25 and 40 µm were selected from each rat to monitor leukocyte-endothelial cell adhesion (rolling and firm adherence), venule diameter, and \( V_{\text{RBC}} \). Quantification of leukocyte-endothelial cell interactions was accomplished through playback of videotaped images. Leukocyte adherence was defined as the number of leukocytes (per 100-µm length of venule) that remained stationary on the vessel wall for a period of at least 30 s during a 2-min observation period. Leukocyte rolling velocity was estimated from the time taken for leukocytes to roll through a 50-µm length of venule (using an average from 10 cells). The number of rolling leukocytes in the 100-µm venule segment at any given time, \( R_{100\mu m} \), was determined from the number of leukocytes that traveled past a selected cross section of venule per minute, \( R_{\text{flux}} \), and the leukocyte rolling velocity, \( V_{\text{RBC}} \) (µm/s): \( R_{100\mu m} = \frac{R_{\text{flux}}V_{\text{RBC}} \times (1 \text{ min/60 s} \times 100 \mu m)}{V_{\text{RBC}} (\mu m/s)} \).
Table 1. Measurements made in young and aged rats before and after ischemia-reperfusion

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Aged</th>
<th>Aged</th>
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<tr>
<td>Blood pressure, mmHg</td>
<td>124 ± 5</td>
<td>112 ± 5*</td>
<td>108 ± 4*</td>
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<tr>
<td>Protein concentration, g/l</td>
<td>51 ± 2</td>
<td>45 ± 3*</td>
<td>57 ± 2</td>
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<tr>
<td>Venular diameter, µm</td>
<td>32 ± 1</td>
<td>32 ± 1</td>
<td>1.5 ± 0.1†</td>
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<tr>
<td>V_RBC, mm/s</td>
<td>2.2 ± 0.3</td>
<td>1.5 ± 0.2*</td>
<td>43 ± 9</td>
</tr>
<tr>
<td>V_WBC, µm/s</td>
<td>66 ± 12</td>
<td>55 ± 10</td>
<td>1.0 ± 0.1†</td>
</tr>
<tr>
<td>R_RBC, min⁻¹</td>
<td>34 ± 6</td>
<td>40 ± 7</td>
<td>1.0 ± 0.1†</td>
</tr>
<tr>
<td>R_WBC, 100 µm⁻¹</td>
<td>1.2 ± 0.2</td>
<td>1.7 ± 0.4</td>
<td>43 ± 9</td>
</tr>
<tr>
<td>Circulating leukocytes, µl⁻¹</td>
<td>8.245 ± 1.022</td>
<td>10.591 ± 1.111</td>
<td>5.227 ± 6.24†</td>
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Data are presented as means ± SE; N = 11 rats in each category except protein concentration (N = 10). Rep-15, Rep-40, 15 and 40 min, respectively, after ischemia-reperfusion; V_RBC, red blood cell centerline velocity; V_WBC, leukocyte rolling velocity; R_RBC, no. of leukocytes rolling past a selected cross section of venule; R_WBC, no. of rolling leukocytes in 100-µm venule segment at any given time. *P < 0.05 compared with baseline; †P < 0.05 compared with corresponding value in young rats.

RESULTS

I/R caused a decline in blood pressure and plasma protein concentration (Table 1) that was similar in young (2–3 mo old) and aged (18–24 mo old) rats. V_RBC in the chosen venules was significantly higher in the younger rats (Table 1), but a similar decline of 40–45% was observed in both age groups by the end of the 40-min reperfusion period.

The higher baseline level of leukocyte rolling (R_WBC) in the aged rats (P = 0.06; Table 1) could possibly be attributed to the increased number of circulating neutrophils in the bloodstream, as shown in Fig. 1. The aged rats had 2,145 ± 348 circulating neutrophils/µl blood compared with 1,191 ± 161 in the younger rats (P < 0.05). This difference was exacerbated after I/R; the aged rats had 7,164 ± 1,060 circulating neutrophils/µl blood compared with 3,218 ± 463 in the younger rats (P < 0.05).

I/R produced a significant increase in the number of leukocytes firmly adhering to the postcapillary endothelium in young rats (Fig. 2): leukocyte adherence increased from a value of 2.8 ± 0.4 per 100 µm (N = 11 rats) during baseline to 6.6 ± 2.0 per 100 µm (P < 0.05) after 15 min of reperfusion and to 6.0 ± 1.6 per 100 µm (P < 0.05) after 40 min of reperfusion. However, no I/R-induced increase in firm adherence was observed in the aged rats (N = 11; P = 0.41), even though approximately the same number of leukocytes were rolling through the venules (see R_WBC in Table 1). The increase in leukocyte adherence in young rats is somewhat lower than that reported by Kurose et al. (14), which can partially be attributed to counting adherence for a period of only 2 min rather than 5 min. (Adherence is defined as the number of leukocytes remaining stationary for at least 30 s during the 2- or 5-min observation period.) Using the shorter period allowed almost simultaneous observation of multiple venules per rat in the current study (2.5 ± 0.2 venules per rat in both young and aged groups).

I/R produced a significant increase in the leakage of FITC-albumin from the postcapillary venules of both young and aged rats (Fig. 3). PI increased from baseline values of 0.08–0.09 to 0.29–0.31 at 40 min of reperfu-

Fig. 1. Number of circulating neutrophils (PMN) in young (N = 11) and aged (N = 11) rats before (Baseline) and after ischemia-reperfusion (Reperfusion). Data are given as means ± SE. *P < 0.05 compared with Baseline; †P < 0.05 compared with corresponding value in young rats.

Fig. 2. Leukocyte adherence in postcapillary venules in young (N = 11) and aged (N = 11) rats before ischemia and 15 and 40 min after reperfusion (Rep-15 and Rep-40, respectively). Data are given as means ± SE. *P < 0.05 compared with Baseline.
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Fig. 3. Permeability index of postcapillary venules in young (N = 11) and aged (N = 11) rats before ischemia and 15 and 40 min after reperfusion. Data are given as means ± SE. *P < 0.05 compared with Baseline.

Fig. 5. Venular shear rates in young (N = 11) and aged (N = 11) rats before ischemia and 15 and 40 min after reperfusion. Data are given as means ± SE. *P < 0.05 compared with Baseline; +P < 0.05 compared with corresponding value in young rats.

Fig. 6. Permeability index (A) and shear rates (B) of postcapillary venules in young (N = 12) and aged (N = 9) rats before (Baseline) and after 30 min of mesenteric superfusion of N\(^{\text{G}}\)-nitro-L-arginine methyl ester (L-NAME). Data are given as means ± SE. *P < 0.05 compared with Baseline.

Fig. 4. Oxidative stress in tissue surrounding postcapillary venules in young and aged rats exposed to either 30 min each of ischemia and reperfusion (solid bars) or an equal time of sham ischemia and reperfusion (open bars). Data are given as means ± SE; N = 5, 7, 5, and 10 rats in groups from left to right. RH-123, rhodamine 123. +P < 0.05 compared with corresponding value in young rats.

I/R produced a significantly higher level (P < 0.05) of oxidative stress in aged rats (N = 10) than in young rats (N = 7), as assessed by the formation of fluorescent RH-123 in the tissue surrounding the postcapillary venules (Fig. 4). One possible reason for the increased oxidative stress in the aged rats is a decreased function of NO, a mediator that is capable of scavenging superoxide produced during reperfusion. The data in Fig. 5 present one reason to suspect a diminished role for NO in the aged rats: venular shear rates are significantly lower (P < 0.05) in aged rats, not only during baseline conditions (289 ± 25 vs. 420 ± 51 s\(^{-1}\) in young rats; N = 11/group) but also after reperfusion. This might be of importance in view of reports of increased endothelial NO synthase levels with increased shear (24). Additionally, plasma cholesterol levels were significantly higher (P < 0.05) in the aged rats (116 ± 12 mg/dl; N = 6) than in the young rats (59 ± 3 mg/dl; N = 5), which is important in view of the possibility of diminished NO function with increasing cholesterol concentration (28).

L-NAME (100 µM) was superfused onto the mesentery to investigate the possibility of a diminished role of NO in the aged rats. As an NO synthase inhibitor, L-NAME is known to produce an increase in postcapillary albumin leakage in young rats (15), an observation that we confirmed in our 2- to 3-mo-old group (see Fig. 6A). However, no increase in PI with L-NAME was observed in the aged rats. This observation would support the premise of a reduced role in the aged rats, because inhibiting NO synthesis might only have an effect where NO function is prominent. Additionally, L-NAME produced a slightly larger decrease (P = 0.08) in venular shear rate in aged rats (74 ± 5%) compared with young rats (59 ± 6%), as shown in Fig. 6B. Blood
pressure remained essentially constant in both age groups, decreasing from $137 \pm 3$ to $133 \pm 4$ mmHg [not significant (NS)] in the young rats and from $121 \pm 4$ to $118 \pm 3$ mmHg (NS) in the aged rats; therefore, the larger decrease in venular shear rate in aged rats was not a secondary effect of a greater decrease in blood pressure. The decline in venular shear rate is largely caused by arteriolar constriction; arteriole diameter was observed to decrease by 5–10% in both young and aged rats. The similarity in the L-NAME-induced arteriolar constriction and decreased shear rates would suggest that NO is functional in the aged rats, at least in the arterioles. However, the greater decline in venular shear rate induced by L-NAME in the aged rats could possibly suggest that the same concentration of L-NAME eliminates a greater proportion of NO synthesis than in the young rats.

**DISCUSSION**

Reperfusion injury has received much attention in the past 15–20 yr. Research into the mechanisms of injury has employed a variety of models in several animal species, most commonly the rat. Although the damage resulting from I/R can affect any age, the elderly are at the greatest risk, and a few recent studies suggest that the response to I/R may differ as a function of age. For example, the outcome of myocardial infarction is more severe in elderly patients (8); studies in rats (17) and rabbits (2) show an age-associated decrease in functional recovery of hearts exposed to I/R, with an increased susceptibility to oxidative injury proposed as a possible mechanism (17). However, despite the obvious need to understand the mechanisms of reperfusion injury in the elderly, virtually all reperfusion research in rats uses young animals. This study begins the important task of determining how the mechanisms of reperfusion injury may be altered with increasing age.

An apparent contradiction in how reperfusion research relates to the elderly is that in young rats, neutrophil-endothelial cell adhesion is considered to be a rate-limiting step in the development of endothelial dysfunction. For example, elimination of circulating neutrophils with anti-neutrophil serum attenuates I/R-induced injury in intestine, skeletal muscle, brain, heart, and liver (10). Furthermore, monoclonal antibodies against the adhesion molecules CD11/CD18 and intercellular adhesion molecule-1, as well as P-, E-, and L-selectin, have been demonstrated to reduce I/R-induced injury (10). The contradiction is that neutrophils in elderly individuals have been shown to have reduced function, including attenuation of 1) chemotaxis (1, 23), 2) phagocytosis (1, 23), 3) bactericidal capacity (23), 4) cytokine release (29), 5) protease release (5), 6) in vitro adherence to endothelium (18), and 7) oxidant production (7, 20). The reasons for impaired neutrophil function have not been well established, but a prominent hypothesis is that the neutrophils of aged individuals experience oxidative damage as a result of an age-associated decline in endogenous antioxidants (4). Therefore, if neutrophils in the elderly are less able to inflict I/R-induced injury, but at least as much (or more) injury occurs in the elderly, then it is possible that I/R-induced injury becomes less neutrophil dependent with increasing age.

One purpose of this study was to compare I/R-induced leukocyte adhesion as a function of age. Our results demonstrated a significant difference between young and old rats (which could possibly be caused by suppressed neutrophil function with increasing age): I/R-induced increases in firm leukocyte adherence occurred only in the young rats. Without an increase in I/R-induced leukocyte adherence in the aged rats, it could be predicted that venular albumin leakage would increase minimally, based on research in 2- to 3-mo-old rats (14), showing that I/R-induced albumin leakage is directly proportional to the number of adherent leukocytes. However, in our study, we found that 30 min of ischemia followed by 40 min of reperfusion resulted in an essentially identical increase in venular albumin leakage in young and aged rats. This finding does not necessarily exclude a role for leukocyte adherence, because two to three adherent leukocytes were present along the selected 100-μm lengths of venule during both the baseline period and the following 40 min of reperfusion. However, the lack of an increase in leukocyte rolling and firm adherence suggests that mechanisms independent of leukocyte-endothelial cell adhesion may have a greater influence in I/R-induced injury in aged rats.

One possible mechanism of I/R-induced injury that may occur independently of leukocyte adhesion is an increase in oxidative stress, which is thought to be more prevalent in the aged. For example, generation of mitochondrial superoxide by rats increases with age (21), and levels of both superoxide and hydrogen peroxide increase with age in the mouse brain, heart, and kidney (25). With respect to antioxidants, investigators have demonstrated age-dependent decreases in superoxide dismutase and catalase (3, 30), as well as indirect evidence for a decrease in endothelial NO (19, 26). In this study, we confirmed an age-associated increase in the level of I/R-induced oxidative stress, as indicated by an enhanced fluorescence of rhodamine 123 in the tissue surrounding the postcapillary venules in the aged rats.

Although the increased oxidative stress might have a number of causes related to the levels of oxidants and/or antioxidants, we sought to investigate whether the role of NO is reduced in the aged rats, based on the following observations. First, there have been reports of a decline in the NO precursors aspartic acid, citrulline, and arginine (27) as well as an age-dependent decrease in endothelium-dependent relaxation (19, 26), which is thought to be mediated by NO. Second, we found a significantly higher concentration of plasma cholesterol in the aged rats; hypercholesterolemia can depress endothelium-dependent relaxation, even before the development of atherosclerosis (28). Third, venular shear rates were significantly lower in the aged rats (before and after I/R); lower shear rates have been
Our experiments with the NO synthase inhibitor L-NAME suggest that NO does remain functional with regard to arteriolar dilation in the aged rats. L-NAME caused a similar degree of arteriolar constriction (and a resultant decrease in venular shear rate) compared with that seen in young rats. In contrast, L-NAME induced an increase in postcapillary albumin leakage only in the young rats. The latter observation can be interpreted in at least two ways, one of which is a decreased role of NO in the aged rats. However, an alternative explanation may be just as likely: if NO function is normal in the aged rats, the lack of an increase in albumin leakage could suggest a leukocyte dysfunction in the aged rats. L-NAME-induced permeability changes are dependent on leukocyte adhesion (9, 12, 15), and if leukocyte function is attenuated in the aged rats, a similar decrease in NO with L-NAME may not have the same leukocyte-mediated effects. However, with either interpretation, data from the L-NAME experiments indicate a fundamental difference between the two age groups in how postcapillary venules respond to this model of acute inflammation.

In summary, this study has demonstrated a number of differences in how young and aged rats respond to I/R. Even though further studies need to be performed to more completely describe how the mechanisms of I/R injury are altered with age, our study suggests that the mechanisms of I/R injury may be sufficiently different in young and old patients to demand differential therapy of clinical I/R based on age.

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