Role of leukocyte accumulation and oxygen radicals in ischemia-reperfusion-induced injury in skeletal muscle

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Schlag, M. G., K. A. Harris, and R. F. Potter. Role of leukocyte accumulation and oxygen radicals in ischemia-reperfusion-induced injury in skeletal muscle. Am J Physiol Heart Circ Physiol 280: H1716–H1721, 2001.—The role of leukocytes and nonleukocyte-derived reactive oxygen metabolites (ROMs) in reperfusion-induced skeletal muscle injury was determined. Male rats received 2 h no-flow hindlimb ischemia-reperfusion (I/R, n = 6) or were rendered neutropenic via antineutrophil serum (ANS) before I/R (n = 5). Oxygen radicals in the absence of neutrophils were tested by administration of dimethylthiourea (DMTU) (I/R + ANS, n = 5). Oxygen radicals in the absence of neutrophils were tested by administration of dimethylthiourea (DMTU) (I/R + ANS + DMTU, n = 5). Perfused capillaries (CDper) and rolling (Lr), adherent (La), and extravasated leukocytes (Le) in the extensor digitorum longus muscle were measured every 15 min during 90 min of reperfusion using intravital microscopy. The vital dyes bisbenzimide (BB) and ethidium bromide (EB) provided direct measures of tissue injury (EB/BB). CDper decreased immediately on reperfusion in the I/R and I/R + ANS groups. CDper in the I/R + ANS + DMTU group remained at baseline throughout reperfusion. Lr increased in the I/R group; however, BB/BB was the same between I/R and I/R + ANS groups. Injury in the I/R + ANS + DMTU group did not differ from other groups ≥60 min, after which BB/BB became significantly lower. Le did not differ between groups and was highly correlated to tissue injury. The results suggest that Le lead to parenchymal injury, and ROMs lead to perfusion deficits during the early reperfusion period after ischemia.

Chronic and acute ischemia in skeletal muscle, particularly in the lower extremities, is a frequent clinical problem and occurs as a consequence of trauma, hemorrhage, vascular stenosis, and thromboembolic events. In most instances surgical techniques allow rapid and successful revascularization. However, clinical and experimental studies have shown significant reperfusion-induced injury as a direct consequence of the restoration of blood flow after ischemia. Such ischemia-reperfusion (I/R)-induced injury has been characterized by edema, impaired blood flow (i.e., microvascular perfusion deficits), muscle injury, and loss of muscle function.

It is generally accepted that polymorphonuclear leukocytes (particularly neutrophils) and reactive oxygen metabolites are key mediators of reperfusion-induced injury in organs such as skeletal muscle. Although the initial reperfusion period has been shown to be decisive for the final extent of reperfusion injury (20), the relative roles of leukocytes and reactive oxygen metabolites remain an active area of study. Use of direct measures of leukocyte-endothelial cell interactions within the microvasculature suggests that significant leukocyte adherence does not occur until 30–45 min after the ischemic period (8, 14, 15). Although such measures may reflect a relatively early time course for leukocyte accumulation, few studies have directly evaluated the correlation between leukocyte accumulation and early reperfusion-induced injury.

Suematsu and colleagues (23) reported that reperfusion-induced myocyte injury after 1 h of hemorrhagic shock occurred before leukocyte accumulation and involved reactive oxygen metabolites via an endothelium-dependent mechanism. However, hemorrhagic shock resulted in a significant increase in leukocyte accumulation within both capillaries and postcapillary venules before the reperfusion phase. Although restoration of the blood volume (thereby returning mean arterial pressure to normal levels) was accompanied by a transient decrease in leukocyte accumulation, the influence of leukocytes before reperfusion remains difficult to assess. More recently, Schlag and colleagues (21) showed that the use of the oxygen radical scavenger dimethylthiourea (DMTU) was efficacious in not only reducing leukocyte-endothelial cell interaction during reperfusion, but also in significantly reducing the incidence of cell injury. However, their study design did not allow for a clear differentiation between the relative roles of leukocytes and reactive oxygen metabolites.

The aim of the present study was to address the relative importance of leukocytes and reactive oxygen metabolites in the early reperfusion period (first 90 min) after 2 h of normothermic no-flow ischemia. To accomplish our goal, we applied intravital microscopy...
to the rat extensor digitorum longus (EDL) muscle, thus allowing for the simultaneous and direct measurement of microvascular blood flow, leukocyte-endothelial interactions, and tissue injury before and after an ischemic insult (6, 18). The role of neutrophils was tested by rendering rats neutropenic before ischemia, and the role of specific oxygen radicals (i.e., hydrogen peroxide and the hydroxyl radical) was determined in the absence of neutrophils by application of the oxygen radical scavenger DMTU to rats rendered neutropenic. Our findings suggested that oxygen radicals but not leukocytes were involved in the microvascular perfusion deficits during the first 90 min of reperfusion after 2 h of no-flow ischemia, although extravasated neutrophils were likely an important mechanism in reperfusion-induced parenchymal injury.

**METHODS**

The experimental protocol was approved by the Council on Animal Care of the University of Western Ontario and has been previously described in detail (5, 6). Briefly, male Wistar rats (weight 220–280 g) were anesthetized with 1–2% halothane, and the left carotid artery and external jugular vein were cannulated to allow for the measurement of systemic blood pressure and fluid administration, respectively. After the skin on the lateral side of the right hindlimb was incised, the biceps femoris muscle was incised to expose the underlying tibialis anterior and gastrocnemius muscles, which were separated. The underlying EDL muscle was dissected free to the level of its distal tendon, which was tied with a suture and cut from its bony insertion. Body temperature was maintained at 37°C with a heat lamp throughout the experiment.

**Intravital microscopy.** After the EDL muscle was prepared, the animal was transferred to the stage of an inverted microscope (Nikon Diaphot 300). The microscope was coupled to a charged-coupled device camera (Dage-MTI VE1000), a video recorder (HR-S10000U VCR, JVC), a time-date generator (WJ-810, Panasonic), and a TV monitor (WV-5410, Panasonic). The EDL was reflected into a saline bath, which was then sealed with a glass coverslip to isolate the EDL from the atmosphere. The muscle temperature was maintained indirectly by shadowing the muscle with aluminum foil and maintaining the bath temperature at 32°C with an infrared lamp. After a 30-min recovery period to allow the microvascular blood flow to stabilize after surgically induced hyperemia, two to three microvascular units consisting of a feeding arteriole, the capillary network, and a draining postcapillary venule were randomly chosen, and the same units were used throughout the study. Thus two to three capillary areas and two to three postcapillary venules were recorded in each EDL muscle before ischemia and at each 15-min time point during reperfusion. We made 1-min video recordings of the capillary network using a ×20 objective and of postcapillary venules using a ×40 objective. There was no spatial overlap between the recorded capillary fields or postcapillary venules. Transillumination was provided by fiber-optic light guides. The baseline values of all parameters except tissue injury were derived from these initial video recordings.

**Ischemia and reperfusion.** After the video recording of the selected fields of view, 2 h of no-flow ischemia was induced by tightening a tourniquet (no. 4 silk) placed around the hindlimb above the greater trochanter. Absence of flow in the EDL during ischemia was determined and continuously monitored using intravital microscopy. The muscle bath was replaced 5 min before reperfusion with saline containing 5 μg/ml each of the fluorescent vital dyes bisbenzimide (BB; excitation 343 nm, emission 483 nm) and ethidium bromide (EB; excitation 482 nm, emission 616 nm). BB stains the nuclei of all cells, but EB stains the nuclei of only those cells with damaged plasma membranes (4). EB/BB, the ratio of EB-labeled nuclei to the total number of nuclei (BB labeled), provided an index of tissue injury (5, 18).

Reperfusion was initiated by removal of the tourniquet, and the previously chosen fields of view were video recorded for 1 min every 15 min throughout 90 min of reperfusion. Microvascular perfusion and leukocytes within postcapillary venules were recorded using transillumination and the ×20 and ×40 objectives, respectively. Fluorescence intravital microscopy was used to identify nuclei stained by EB and BB from the same fields of view that had been selected for the measurement of capillary perfusion. For fluorescence microscopy the ×20 objective was used, and the preparation was exposed to the appropriate excitation wavelengths (100-W mercury lighthouse, model HB-10101AF, Nikon) for no more than 5 s in each field of view. Rats were euthanized at the end of the experiments by an overdose of anesthetic agent.

**Experimental groups.** Rats were randomly allocated to one of three experimental groups: I/R (n = 6), I/R + antineutrophil serum (I/R + ANS, n = 5), and I/R + ANS + DMTU (n = 5). Rats in the I/R + ANS and I/R + ANS + DMTU groups were rendered neutropenic by an intraperitoneal injection of ANS (Accurate Chemical and Scientific) in isotonic saline (1 ml, 1:5 ANS-to-saline ratio) after cannulation of the carotid artery, and I/R rats received an equivalent volume of saline. We previously showed that this dose of ANS results in profound neutropenia within 1 h (6). Arterial blood samples (250 μl) were taken before injection of ANS or vehicle (baseline), after 1 h of ischemia, and at 90 min of reperfusion for total and fractional white blood cell counts. The withdrawn blood volumes were replaced with saline. Rats in the I/R + ANS + DMTU group received a bolus of DMTU (1 g/kg; 25%) before reperfusion. Animals in the I/R and the I/R + ANS groups received a volume-matched bolus of vehicle (isotonic saline). Bolus injections were given intravenously over a 2-min period starting 10 min before reperfusion.

**Off-line video analysis.** Capillary perfusion was assessed by counting the number of perfused capillaries (CD_{per}; i.e. capillaries with flowing red blood cells) that crossed three nuclei, EB/BB. The estimate of injury was then expressed as the ratio of EB- to BB-labeled nuclei, EB/BB.

The numbers of rolling (L_{r}) and adherent leukocytes (L_{a}) in postcapillary venules were measured and expressed per 1,000 μm^2. A leukocyte was considered stuck to the wall of the venule if it remained stationary for ≥30 s, and a cell was considered rolling if it remained in contact with the wall of the vessel during its movement. Very small and dense-bodied leukocytes were identified as lymphocytes and were excluded. Extravasated leukocytes (L_{e}) were counted within an area equal to 1,000 μm^2 adjacent to the postcapillary venule.

**Statistical analysis.** All data are presented as means ± SE if not stated otherwise. Preischemic values were compared using one-way ANOVA. One-way repeated measures ANOVA was used for comparisons within groups, and two-way repeated measures ANOVA was used for comparisons between groups. To isolate individual time points or treatments, ANOVA was followed by Dunnett’s test or a Student-
Newman-Keuls test. Statistical significance was accepted at an error level of $P \leq 0.05$. All statistics were undertaken using SigmaStat 1.0 (Jandel).

**RESULTS**

The average mean arterial pressure (MAP) in the I/R group (94.1 ± 0.5 mmHg) was significantly higher than in the I/R + ANS and I/R + ANS + DMTU groups (91.6 ± 0.7 mmHg and 89.5 ± 0.9 mmHg, respectively). However, all pressures were within an accepted physiological range for rats. With the exception of the I/R + ANS + DMTU group, MAP did not vary during the entire experimental period. However, the administration of DMTU caused a significant albeit transient (2–3 min) decrease in MAP from 93.4 ± 1.2 to 77.8 ± 2.4 mmHg.

The baseline numbers of circulating neutrophils did not differ between groups (Fig. 1). The rats in the I/R + ANS and the I/R + ANS + DMTU groups showed profound neutropenia during I/R (Fig. 1), although the I/R rats had significantly increased numbers of circulating neutrophils at the two later sampling times compared with baseline values.

**Capillary reperfusion.** The preischemic values of $C_{per}$ did not differ between groups (31.1 ± 1.2 for I/R, 31.3 ± 1.8 for I/R + ANS, and 30.1 ± 1.1 for I/R + ANS + DMTU). In the I/R and the I/R + ANS groups, reperfusion was accompanied by a significant decrease in $C_{per}$ that persisted throughout reperfusion (Fig. 2). Neutropenia alone had no beneficial effect on capillary perfusion, although $C_{per}$ in the I/R + ANS + DMTU group recovered to preischemic levels after an initial significant decrease (Fig. 2). In the I/R + ANS-treated group, reperfusion began (0 min) with a highly significant decline in $C_{per}$. The mean $C_{per}$ value at this time of reperfusion was highly variable owing to one rat that showed complete albeit transient cessation of flow after a brief initial period of reperfusion. $C_{per}$ during reperfusion was significantly higher in the I/R + ANS + DMTU group than in the I/R group at all reperfusion times and at 15, 75, and 90 min of reperfusion compared with the I/R + ANS group.

**Tissue injury.** The index of tissue injury increased progressively reaching a constant level within 60–75 min of reperfusion in all groups. The average tissue injury was not statistically different between the I/R and the I/R + ANS groups throughout reperfusion (Fig. 3). In contrast, a trend toward reduced tissue injury occurred throughout reperfusion in the I/R + ANS + DMTU group.
DMTU group, which became significantly different from the other two groups within 75 min of reperfusion (Fig. 3).

Leukocyte behavior. The preischemic values of \( L_r, L_a, \) and \( L_e \) in the I/R, I/R + ANS, and I/R + ANS + DMTU groups were not significantly different. In the I/R group, \( L_r, L_a, \) and \( L_e \) increased progressively during reperfusion; however, in the neutropenic groups, \( L_r, L_a, \) and \( L_e \) remained at preischemic values throughout reperfusion (Fig. 4). There were no significant differences in the average \( L_e \) between all groups throughout reperfusion (Fig. 4C).

Leukocyte-endothelial cell interaction within the microvasculature is an essential step in the inflammatory process. However, it is likely that the cells most important for parenchymal injury are those that had extravasated. Thus in the I/R group there was a strong linear correlation (\( r^2 = 0.97 \)) between the numbers of \( L_e \) and tissue injury throughout the 90-min reperfusion period (Fig. 5).

DISCUSSION

Neutropenia or antibodies against adhesion molecules have often been used to define the role of leukocytes in I/R-induced injury to the microvasculature and parenchyma (1, 3, 6, 9, 11, 16, 20, 24). In addition, oxygen radical scavengers have been shown to reduce leukocyte-mediated injury (7, 21) and improve microvascular perfusion and tissue injury after ischemia (21). However, the relative roles played by leukocytes and reactive oxygen metabolites during the early reperfusion phase after ischemia has not been as extensively studied.

In the present study, leukocyte adhesion increased significantly within the microvasculature after 60 min of reperfusion, which was after 2 h of normothermic no-flow ischemia in the EDL muscle. On the basis of direct measures of leukocyte-endothelial cell interaction, such results agree closely with previous studies using either 1 h (23) or 2 h (8, 14) of ischemia. However, the results of these studies are at variance with those using indirect or whole organ estimates. For example, Söjö and co-workers (22) found no increase in the level of myeloperoxidase (MPO) from the rat hindlimb after 12 h of reperfusion after 1.5 h of no-flow ischemia. If the severity of the ischemic event were increased to 3 h, significant leukocyte accumulation was noted after 5 h of reperfusion. Similarly, Rubin and co-workers (20) showed only slight increases in MPO activity during the first 3 h of reperfusion; however, MPO levels were elevated ~105-fold after 48 h, which was after 5 h

Fig. 4. Numbers of rolling (A), adherent (B), and extravasated (C) leukocytes per 1,000 \( \mu \)m\(^2\) were measured before ischemia (Pre-I) and every 15 min of reperfusion (R). Leukocyte rolling increased immediately on reperfusion (time 0) after limb ischemia (○); however, no change occurred with I/R + ANS (●) or I/R + ANS + DMTU (▲) treatments. Significantly increased leukocyte adhesion required 60 min of reperfusion, although only 30 min was required for significantly increased extravasation. *\( P \leq 0.05 \) compared with time 0; †\( P \leq 0.05 \) compared with 60 min time.

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of no-flow ischemia. Such differences in the time course of leukocyte accumulation likely reflect the sensitivity of the methodologies and/or the models used. Thus results based on direct measures of leukocyte-endothelial cell interaction suggest that leukocytes may become involved in reperfusion-induced injury much earlier than would have been predicted from indirect measures.

In a previous study using exactly the same preparation and methodology (21), we demonstrated that the background level of tissue injury (EB/BB) within the naive EDL (no I/R) was 0.29 ± 0.01 and did not significantly change during the duration of the 90-min observation period. Compared with this background level of tissue injury, all the experimental groups in the present study had significantly elevated tissue injury within 15–30 min of reperfusion. Although it may be tempting to speculate that the I/R + ANS + DMTU group had less ischemic-induced injury based on what appeared to be a trend toward more injury in the I/R and I/R + ANS groups, no significant differences were measured between all groups at the onset of reperfusion.

The present study provides evidence of tissue injury at a time when leukocyte adhesion within the microvasculature had not yet reached significantly increased levels. Although such results are in agreement with those of Suematsu and colleagues (23), measures of leukocytes within the microvasculature may be misleading because the Le would be more closely linked to myocyte injury. Such a hypothesis would be supported by the observations that 1) reduced leukocyte adhesion (via ANS-induced neutropenia) did not lead to a significant reduction in tissue injury, and 2) a strong linear correlation exists between the numbers of Le and tissue injury throughout the 90-min reperfusion period (Fig. 5). Interestingly, use of the oxygen radical scavenger DMTU in the absence of neutrophils was more effective in altering reperfusion-induced injury to the parenchyma than in reducing the microvascular accumulation of leukocytes per se. Because the potential for leukocyte-derived reactive oxygen metabolites is a generally recognized mechanism leading to reperfusion injury, it is tempting to suggest that activation of resident Le represents an important factor in the early reperfusion-induced injury measured in the present study.

DMTU is a highly effective scavenger of hydrogen peroxide and hydroxyl radicals (7, 10) and has been used in vitro and in vivo to attenuate oxidant stress and I/R-mediated injury (2, 7, 12, 21). It has been suggested that the administration of oxygen radical scavengers will reduce but not prevent leukocyte accumulation during reperfusion (13). However, we showed (21) that the use of DMTU in the same experimental model used in the present study resulted in a significant reduction in leukocyte adhesion, and the number of Le remained at preischemic levels throughout reperfusion. Although the use of such treatment significantly reduced microvascular perfusion and tissue injury, we were unable to conclude which change was the more important: reduced leukocyte accumulation, scavenging reactive oxygen metabolites, or both. Therefore, in the present study, we combined the treatments (ANS + DMTU) in addition to separately testing the effect of ANS alone. We believe that the use of such a protocol allowed us to better test the individual role of leukocytes and nonleukocyte-derived oxygen radicals as mechanisms leading to tissue injury and microvascular perfusion deficits after ischemia. Whereas treatment with ANS alone had no beneficial effect, the additional administration of DMTU prevented capillary no-reflow following ischemia. Such results suggest that the initial capillary malperfusion after a short ischemic event was mediated via oxygen radicals rather than leukocyte plugging of the microvasculature. The mechanism whereby reactive oxygen-mediated injury may lead to altered capillary perfusion was not tested in the present study. However, it is conceivable that loss of volume regulation may result in endothelial cell swelling thereby increasing resistance to blood flow. Such a hypothesis requires further investigation.

The lag between increased leukocyte adhesion (60 min) compared with extravasation (30 min) in the I/R group may reflect a significant increase in the rate of extravasation. However, it is important to note that there was no significant difference in the numbers of Le throughout reperfusion between I/R and the two neutropenic groups. Although the similarities in the magnitude of Le between groups might appear surprising, such might be expected based on the use of ANS. ANS treatment reduces the circulating leukocyte level thereby generating neutropenia by causing leukocyte sequestration within tissues. We had not expected ANS treatment to result in significant extravasation within skeletal muscle, but the fact that similar levels of Le were present in the tissue during reperfusion in all groups taken together with the fact that leukocyte extravasation was highly correlated to tissue injury may help to explain why there was no difference in injury in the I/R + ANS compared with the I/R groups. Thus we believe that activation of resident extravascular leukocytes together with the increase in leukocyte extravasation that occurs after 30 min of reperfusion likely explains the onset and progressive increase in parenchymal injury.

The degree of reperfusion-induced injury is believed to increase in proportion to the ischemic duration (8) with leukocyte recruitment accepted as one mechanism leading to such injury. However, Suematsu and co-workers (23) were unable to demonstrate a correlation between the temporal development of muscle cell injury and leukocyte accumulation during the early reperfusion phase after 1 h of ischemia. Forbes and colleagues (6) showed that neutropenia prevented the no-reflow phenomenon and significantly reduced tissue injury during the first 90 min of reperfusion after 3 h of no-flow ischemia in the EDL muscle. Similarly, Carden and co-workers (3) demonstrated that the no-reflow phenomenon and the increase in microvascular permeability after 30 min of reperfusion, which was after 4 h
of no-flow ischemia in skeletal muscle, was neutrophil mediated. Because the duration of the ischemic period in the latter two studies (3 or 4 h) was considerably longer than in the present study (2 h) or in Suematsu's study (1 h of hemorrhagic shock), the degree of neutrophil involvement in the early reperfusion injury likely depends on the severity of the ischemic event.

Differentiation of endothelial and myocyte nuclei has often been based on nuclear morphology. In the present study, we were often able to distinguish myocyte and endothelial cells based upon such differences in the shape of the nuclei. However, such differentiation was difficult due to the relatively smaller nuclei of the endothelial cell and the density of myocyte nuclei. We believed that any attempt to differentiate the injury to endothelial cells versus myocytes would result in a significant underestimate of the endothelial cell population. Thus we made no attempt in the present study to differentiate different cell populations. Future studies may find that intravascular application of fluorescent dyes may prove to be a valuable tool in distinguishing these cell types, because endothelial cells may be stained before the surrounding tissue (19).

In conclusion, reactive oxygen metabolites but not neutrophils appear to contribute to the onset of microvascular perfusion deficits associated with the early reperfusion phase after 2 h of normothermic no-flow ischemia of rat skeletal muscle. The strong correlation between leukocyte extravasation and tissue injury during reperfusion suggests that activation of resident leukocytes may be involved in reperfusion-induced tissue injury. Whether reactive oxygen metabolites from nonleukocyte sources activate the resident extravascular leukocytes during the early stages of reperfusion remains to be tested.

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