Ischemia Activates Neutrophils but Inhibits Their Local and Remote Diapedesis

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Hindlimb ischemia and reperfusion results in local limb and distant lung injury. This study tests whether the mechanism of injury is by ischemia mediated polymorphonuclear leukocyte (PMN) activation and diapedesis. Anesthetized rabbits were subjected to three hours of hindlimb ischemia (n = 8) or sham ischemia (n = 4). PMN derived solely from the reperfused ischemic limb and assayed flow cytometrically displayed an oxidative burst of 135 ± 8 fentamoles dichlorofluorescein (fmDCF)/cell compared to preischemic levels of 74 ± 14 fmDCF/cell ($p < 0.05$). Additional aliquots of isolated neutrophils were treated with phorbol myristate acetate (PMA) 10^{-7}M. In contrast to a 162% increase in oxidative burst before ischemia, neutrophils at ten minutes of reperfusion had an enhanced response to PMA of 336% ($p < 0.05$). Plasma collected from the ischemic hindlimb at ten minutes of reperfusion when introduced into an abraded skin chamber or intratracheally induced diapedesis in nonischemic animals. PMN accumulations in the skin chamber were 1636 ± 258 PMN/mm^3 after three hours (n = 8) compared to 63 ± 18 PMN/mm^3 induced by sham plasma (n = 4, $p < 0.05$). Introduction of ischemic plasma intratracheally into a lobar bronchus (n = 4) induced PMN accumulations after three hours, measured by bronchoalveolar lavage fluid of 19 ± 2 × 10^4 PMN/mm^3 compared to 5 ± 1 × 10^4 PMN/mm^3 with sham plasma (n = 4, $p < 0.05$). Diapedesis was completely prevented (0–3 PMN/mm^3, $p < 0.05$) by introducing ischemic plasma into skin chambers in animals whose hindlimbs had been made ischemic (n = 6) or into chambers located on skin regions that had been previously made ischemic (n = 6). Similarly after hindlimb ischemia, lavage of the lung with ischemic plasma yielded few PMN 0–3/mm^3 ($p < 0.05$). These data indicate that ischemia and reperfusion lead to generation of a circulating component in plasma that causes an oxidative burst in PMN and inhibits their diapedesis but promotes diapedesis when applied extravascularly to a naive animal.

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ISCHEMIA AND REPERFUSION induce polymorphonuclear leukocyte (PMN)-dependent tissue injury. Thus following lower torso ischemia, there is a prominent accumulation of leukocytes in, but not outside of, the pulmonary microvasculature.[1] Neutrophils are not seen in the lung interstitium and cannot be recovered by bronchoalveolar lavage (BAL). Furthermore neutrophils are not found with light microscopy in the tissue of the reperfused lower torso. Despite the physical absence of neutrophils in the interstitium it is believed that these cells mediate increased microvascular permeability.[2] We postulate that the reperfused tissue generates a factor(s) that enhances PMN activation, induces PMN adhesion in the microcirculation, but inhibits local and remote diapedesis.

To test this hypothesis, the PMN oxidative burst was assayed using flow cytometry after hindlimb ischemia. Furthermore plasma collected during reperfusion was introduced into abraded skin chambers or intratracheally into nonischemic animals to measure its effects on diapedesis. The same studies were conducted in ischemic animals to evaluate possible inhibition of PMN accumulations.

Materials and Methods

Animal Preparation

Forty-seven New Zealand white male rabbits weighing 3 to 6 kg were used. Initial anesthesia was achieved with intramuscular ketamine (35 mg/kg) and intravenous xy-

lazine (5 mg/kg) and maintained with xylazine 2 mg/kg every 30 minutes. Saline, 0.3 mL/kg/hour was infused *via* a carotid arterial cannula placed aseptically *via* a small neck incision on the day of the experiment. All animals were placed on 37 C heating pads.

Hindlimb Ischemia

Anesthetized rabbits (n = 8) underwent three hours of bilateral hindlimb tourniquet ischemia. Cuffs were inflated to 160 mmHg. Before completion of the ischemic period, the vena cava was ligated just above the iliac confluence to ensure collection of blood derived solely from the ischemic region. Activated neutrophils are known to sequester in the lung microcirculation and if sampling were conducted from the systemic circulation might be unavailable for sampling. Moreover lung leukosequestration after reperfusion induces eicosanoid generation. This methodology excludes nonischemic tissue-derived metabolites. During the first ten minutes of reperfusion, the venous return of both hindlimbs was collected from the vena cava, distal to its point of ligation (approximately 0.5 mL/minute) and discarded. A similar volume of saline was simultaneously replaced *via* the carotid line. In previous studies by our laboratory, it was found that plasma thromboxane B_2 and leukotriene B_4 levels peaked ten minutes after reperfusion of the ischemic hindlimb, whereas immediate levels were not raised. Therefore after the ten-minute washout, 4 mL of hindlimb venous return was collected in cooled heparinized syringes containing 0.1 mL ethylene diamine tetracetic acid (0.07 mg/mL) and aspirin (0.05 mg/mL) and transferred on ice for flow cytometry. Thereafter another 10-mL sample of blood was collected in a similar manner and centrifuged at 1500 $\times g$ for 20 minutes. Aliquots of 0.5 mL plasma were frozen at −20 C and subsequently used in an *in vivo* chemotactic assay. Previous studies have shown that heparin, EDTA, or aspirin in doses used in plasma did not interfere with the chemotactic assay.

Sham Rabbits

Animals (n = 4) were prepared as above but were not subjected to ischemia.

Flow Cytometry

Intracellular generation of H_2O_2 by blood neutrophils was quantitated using flow cytometry and dichlorofluorescein-diacetate (DCFH). DCFH is a nonfluorescent compound that is oxidized to the highly fluorescent dichlorofluorescein (DCF) within neutrophils undergoing a respiratory burst. Leukocytes were isolated from blood using dextran sedimentation, 6% in 0.9% saline, (0.3 mL dextran solution per 3 mL blood) for 45 minutes at room temperature (Dextran T 500, Pharmacia, Piscataway NJ). In preliminary experiments, it was found that any centrifugation, vortexing, or even vigorous pipetting led to increases in baseline oxidation in neutrophils, so these procedures were eliminated in the final protocol. Aliquots of leukocyte-rich sediment (0.01 mL) were added to 1 mL of balanced salt solution (BSS). This solution contained NaCl (124 mM), KCl (5.8 mM), dextrose (10 mM), and hydroxyethylpiperazine ethanesulphonic acid (20 mM) and was titrated with NaOH to pH 7.4 before use. The BSS also contained DCFH (Molecular Probes, Eugene OR) and either buffer or phorbol myristate acetate (PMA) 10^{-7}M. The concentration of DCFH was 100μM, an amount that saturated leukocytes in samples from sham or experimental animals. After incubation for 20 minutes at 37 C the samples were placed on ice and analyzed with an Ortho Diagnostics System 2151 Cytofluorograf flow cytometer using the 488 ηm excitation line of an argon laser at 125 mw output. The PMN within each sample were identified by light scattering. After electronic gating, the green fluorescence of these cells in unstimulated and PMA-stimulated samples was quantitated (3000 to 5000 neutrophils per sample). In some experiments, the fluorescence values obtained by flow cytometry were calibrated with samples of pure rat neutrophils. These cells were obtained four hours after intraperitoneal glycogen. The cells were suspended in BSS, 10^6 cells/mL, and labeled with DCFH with or without PMA treatment. Measurements were conducted in a fluorometer (Perkin-Elmer, Norwalk, CT). Using a standard curve constructed with reagent grade DCF (Sigma Chemical, St. Louis, MO) the amount of DCFH oxidized to DCF by neutrophil H_2O_2 was quantitated, allowing conversion of the mean fluorescence channel number to fentamoles (fm) of DCF produced per cell, a value equivalent to fm of H_2O_2 produced per cell.

Skin Abrasion Blister Chambers

Chemotactic responses were measured by a modification of the technique of Otani.[3] After anesthesia, a 20 × 25 cm area of the back of the rabbits was clipped with electric shears (Wahl Clipper Co., Sterling, IL). These animals (n = 24) were only used to assay chemotaxis. The clipped region was coated with sodium thioglycolate (Lemon Scented Nair, Carter Products, New York, NY) for 15 minutes, washed with tap water, rinsed with 100 mL of 0.25% acetic acid, and then rerinsed with tap water. The animals were permitted to rest for 24 to 36 hours to allow any nonspecific inflammatory response to subside before chemotactic studies were performed. Animals exhibiting skin sensitivity were excluded from the study.

On the day of the experiment, the animal was reanesthetized and a circular area of hair-free skin was outlined

with a template having a 9/32 inch diameter (Rapi-Design Template No. 40, Rapi-Design Products, Burbank, CA). This area was gently abraded with an electric ink eraser (Petty Electric Ink Eraser, Pierce Corp., River Falls, WI) until uniform glistening was produced. This normally took 15 to 20 seconds. The debris from the abraded area was removed by pressing adhesive tape (Blender M, 3M Surgical Products, St. Paul, MN) over the site. On rare occasions, when abrasion led to trace bleeding, the site was abandoned.

Plastic, unit-dose blister chambers (Rexhaus Corp., Westfield Industrial Park, Westfield, MA) with a volume capacity of 0.5 mL were placed over the abraded areas and secured with Steridrape (3M Surgical Products, St. Paul, MN). Usually 18 to 24 blister chambers could be affixed to each rabbit. Injections into the chambers were made with a 27-gauge needle. At the conclusion of the experiment, fluid was withdrawn from the chambers and neutrophils were counted with a hemocytometer. Animals were allowed to rest for 14 days before they were used again.

Plasma Aspiration

A tracheostomy was performed with a 7-mm tube. Through this tube a fine polyethylene cannula (internal diameter, 0.64 mm Delmed, Inc., Canton, MA) was introduced into the bronchus of the middle lobe of the right lung. Evans Blue dye 0.2 mg was added to the lavage solution for later confirmation of the location of the fine cannula. One milliliter of ischemic or normal plasma was introduced *via* the cannula into the bronchus of the middle lobe of the right lung and after five minutes the cannula was removed. Three hours later the animals were killed with an overdose of ketamine. A thoracotomy was performed and the left lung bronchus clamped. Bronchoalveolar lavage (BAL) was performed *via* the tracheostomy tube using three applications of 5 mL of saline. The BAL recovery of about 10 mL was centrifuged at 6000 rpm for five minutes (GCL-1 Centrifuge, Sorvall, Newton, CT). The pellet was suspended in 1 mL saline and PMN counted after Diff-Quik staining to identify macrophages (AHS del Caribe, Inc, Aguada, Puerto Rico).

Skin Capillary Blood Flow

This was measured using laser doppler (Laserflow Model BPM, No. 3, TSI, Inc., St. Paul, MN). The doppler signal produced an output proportional to blood flow, expressed in mL/100 g/minute.[4]

Experimental Protocol

Anesthetized rabbits were subjected to bilateral hindlimb ischemia (n = 8) or sham ischemia (n = 4) for three hours. After ten minutes of reperfusion the venous effluent was collected. PMN were separated and used for flow cytometry and the plasma used for blister and intratracheal treatments. The plasma was introduced into blisters on the animals whose hindlimbs had been made ischemic (n = 9), who had been subjected to sham ischemia (n = 8), or whose dorsal skin used for the blister preparation had been made ischemic with a Satinsky clamp (n = 6). Plasma collected from animals who had undergone sham ischemia was used as controls in all studies. Hindlimb or skin ischemia was for a period of three hours. Ten minutes of reperfusion was allowed before plasma was placed in the blister pack. In lung diapedesis studies, ischemic or sham plasma was introduced into the bronchus of the right middle lobe of animals subjected to three hours of hindlimb ischemia and ten minutes of reperfusion (n = 8) or sham ischemia (n = 4).

Data are expressed as mean ± SEM in text and figures. An analysis of variance, followed by a nonpaired Student's t test was used to determine significance between groups. Significance was accepted if p was less than 0.05.

Animals in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education and Welfare, Publication No. 78-23 [National Institute of Health], revised, 1978).

Results

Neutrophils derived from the reperfused hindlimb displayed an oxidative burst of 135 ± 8 fmDCF/cell compared to preischemic levels of 74 ± 14 fmDCF/cell ($p < 0.05$). Additional aliquots of isolated neutrophils treated with PMA 10^{-7}M showed a 162% increase in H_2O_2 production before ischemia and an enhanced response of 336% after ischemia ($p < 0.05$, Fig. 1). Ischemic plasma, when introduced into abraded skin chambers or intratracheally, induced diapedesis in nonischemic animals. There were 1636 ± 258 PMN/mm^3 in the skin chambers after three hours compared to 63 ± 18 PMN/mm^3 induced by sham plasma ($p < 0.05$, Fig. 2). Introduction of ischemic plasma into the lung of nonischemic animals yielded, by BAL, accumulations of $19 \pm 2 \times 10^4$ PMN/mm^3 compared to $5 \pm 1 \times 10^4$ PMN/mm^3 induced by sham plasma ($p < 0.05$). In animals subjected to three hours of hindlimb ischemia and ten minutes of reperfusion, the ability of ischemic plasma to induce diapedesis in skin blisters or lungs was abolished. The recovery of PMN was 0–3/mm^3 in blister fluid and BAL ($p < 0.05$). Finally ischemic skin rendered prevented diapedesis induced by ischemic plasma introduced into blisters on the previously ischemic skin (0–3 PMN/mm^3, $p < 0.05$).

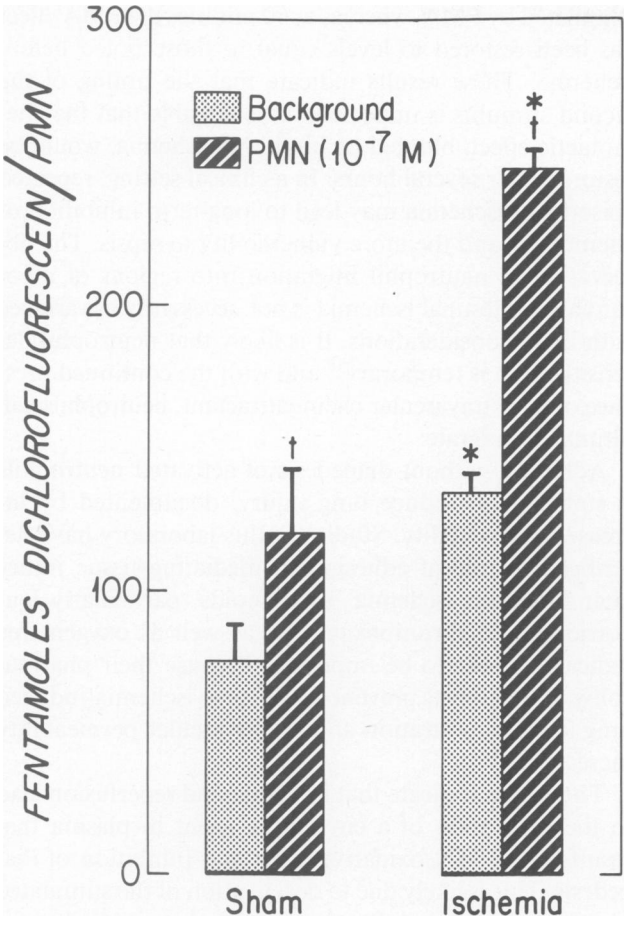

FIG. 1. Neutrophil H_2O_2 production measured flow cytometrically was enhanced following ischemia and by treatment with phorbol myristate acetate. Asterisks and daggers refer to significance ($p < 0.05$) between and within groups.

Skin Blood Flow

Baseline skin blood flow, measured with the laser doppler was 5.6 ± 0.18 mL/100g/minute. During hindlimb ischemia the dorsal abraded skin region showed increased blood flow of 6.8 ± 0.25, mL/100g/minute ($p < 0.05$). In one hour it had returned to baseline levels of 5.4 ± 0.14 mL/100 g/minute. During skin ischemia flow was reduced to 0.04 ± 0.01 mL/100 g/minute while after one hour of reperfusion flow increased to 6.4 ± 0.2 mL/100 g/minute ($p < 0.05$) and then returned to the preischemic levels of 5.7 ± 0.3 mL/100 g/minute.

Discussion

Ischemia and reperfusion lead to PMN-dependent injury. Recent studies have emphasized the role of PMN adhesion in modulating injury after reperfusion. Thus leukocyte-adherence receptor antibodies prevented adhesion and subsequent injury.[5,6] We postulate that ischemia leads to PMN activation, manifest by an oxidative burst and adherence receptor upregulation and that both phenomena mediate the reperfusion injury.

The first part of this study was designed to test the ability of reperfused tissue to increase PMN oxidative activity. Flow cytometry of PMN derived from the venous effluent of reperfused tissue demonstrated an increase in H_2O_2 production as well as an exaggerated response to a second stimulus, PMA. The mechanism of ischemia-induced PMN activation is unknown but may be *via* arachidonic acid derivatives. Thus leukotriene (LT) B_4 synthesized by reperfused tissue is a chemoactivator.[1] By stimulating a receptor site on the PMN surface, activation and an oxidative burst can ensue. In addition LTB_4 may stimulate leukocyte-adherence receptor expression.[7]

The second part of the study tested the ability of ischemic plasma to induce diapedesis. It was found that plasma derived from the ischemic hindlimb during reperfusion induced diapedesis in both abraded skin chambers as well as in the lungs of nonischemic animals. These data suggest that reperfused tissue generates a factor(s) that increases PMN–endothelial interaction. The ability of ischemic plasma to induce diapedesis when applied extravascularly may also be leukotriene dependent. Thus ischemic plasma contains increased levels of LTB_4, which is known to be both a powerful chemoactivator and chemoattractant.

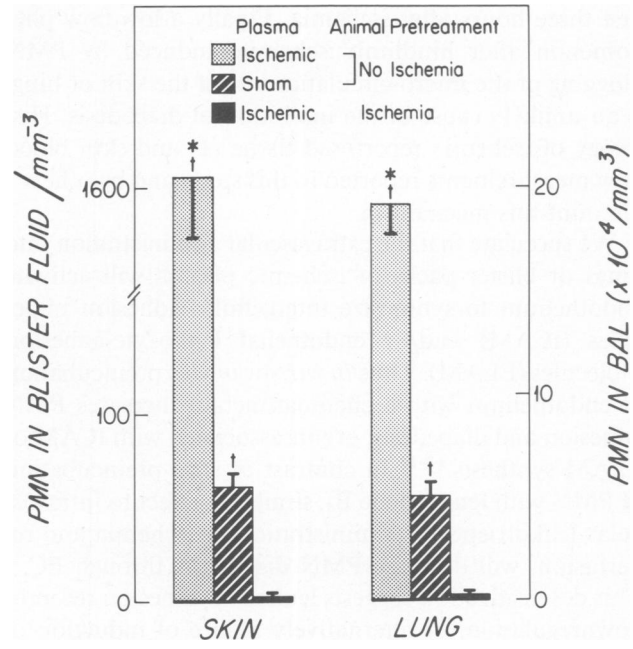

FIG. 2. PMN counts in skin blisters and bronchoalveolar lavage fluid were increased by ischemic plasma relative to sham plasma in animals that had not been made ischemic ($p < 0.05$) as indicated by asterisks. In contrast, when animals were subjected to hindlimb ischemia, diapedesis into skin blisters or lung was prevented even compared to sham plasma ($p < 0.05$) as indicated by daggers.

The third part of the study tested the effect of local and remote ischemia in moderating diapedesis. Surprisingly, after ischemia of skin, ischemic plasma with a known chemotactic ability could not induce diapedesis when instilled into blister chambers positioned on this previously ischemic skin region. Similarly, after hindlimb ischemia, ischemic plasma introduced by lavage into the lung or into the blister packs failed to induce diapedesis. It has been reported that mixing PMN with a chemoattractant will inhibit subsequent directed migration.[8] This is consistent with our observations that the intravascular administration of a chemoactivator induced by ischemia and reperfusion will inhibit a later chemotactic stimulus. These results suggest a desensitization of the neutrophil. Other interpretations are possible. Thus chemoactivators released intravenously may stimulate endothelium to synthesize prostaglandins that could serve as inhibitors of PMN adhesion and diapedesis. This is unlikely because neither indomethacin nor aspirin pretreatment of endothelial cells, to inhibit prostaglandin synthesis, prevented neutrophil adhesion.[9] Also, activated endothelial cells may generate a noncyclooxygenase-dependent leukocyte adhesion inhibitor.[10] Reports indicate that such an inhibitor acts directly on the leukocyte and is blocked by actinomycin D but not by indomethacin or aspirin. However it is unlikely that ischemic tissue synthesizes a leukocyte adhesion inhibitor because the time scale of synthesis of this stable protein is four hours.[10] In our study there was complete inhibition of diapedesis over the course of the first three hours after ischemia. Finally a low-flow phenomenon after hindlimb ischemia, induced by PMN plugging of the micro-circulation[11,12] of the skin or lung, is an unlikely cause of the inhibition of diapedesis. Histology of ischemic reperfused tissue (1) and skin blood flow measurements reported in this study and by others[13] discount this mechanism.

We speculate that the extravascular administration into lungs or blister packs of ischemic plasma will activate endothelium to synthesize intercellular adhesion molecules (ICAM) and/or endothelial leukocyte-adhesion molecules (ELAM). Thus *in vitro* or *in vivo* preincubation of endothelium with a chemoattractant increases PMN adhesion and diapedesis, events associated with ICAM or ELAM synthesis.[14–17] In contrast *in vitro* preincubation of PMN with leukotriene B_4, similar in effect to intravascular leukotriene B_4 administration or ischemia and reperfusion, will decrease PMN diapedesis through EC.[18] This desensitization suggests leukocyte adhesion receptor downregulation, or alternatively failure of induction of endothelial ICAM or ELAM. A recent study has described the sequence of neutrophil activation after hindlimb ischemia.[19] The oxidative burst and prominent additional response to PMA stimulation are noted after five minutes. At 30 minutes of reperfusion the neutrophils cannot be stimulated by PMA, whereas at 60 minutes the PMA effect has been restored to levels equal to those noted before ischemia. These results indicate that the timing of the second stimulus is important. It is possible that the chemotactic effect, blunted by hindlimb ischemia, would be restored after several hours. In a clinical setting, repeated episodes of ischemia may lead to long-term inhibition of chemotaxis and therefore vulnerability to sepsis. The observation of neutrophil migration into regions of myocardial or intestinal ischemia is not necessarily at variance with these considerations. It is likely that neutrophil desensitization is temporary[18] and with the continued presence of an extravascular chemoattractant, neutrophils will ultimately migrate.

Adhesion without diapedesis of activated neutrophils is sufficient to induce lung injury, documented by increased permeability. Studies in this laboratory have described the role of adhesion in mediating tissue injury after hindlimb ischemia. Eicosanoids, particularly leukotrienes and thromboxane A_2, as well as oxygen free radicals, appear to be important because their pharmacologic antagonists prevent lower-torso ischemia-induced lung leukosequestration and microvascular permeability increase.[20–24]

These data indicate that ischemia and reperfusion lead to the generation of a circulating agent in plasma that results in a PMN oxidative burst and inhibition of diapedesis. This is likely due to deactivation of the stimulated neutrophil because ischemic plasma, when applied extravascularly, enhances PMN migration.

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