Mechanisms responsible for enhanced inflammatory response to ischemia-reperfusion in diabetes

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Salas, Azucena, Julián Panés, J. Ignasi Elizalde, Maria Casadevall, Donald C. Anderson, D. Neil Granger, and Josep M. Piqué. Mechanisms responsible for enhanced inflammatory response to ischemia-reperfusion in diabetes. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1773–H1781, 1998.—The objective of the present study was to assess the role of lipid mediators and adhesion molecule expression in exacerbation of ischemia-reperfusion-induced inflammatory response in diabetes. Leukocyte-endothelial cell interactions were studied in mesenteric venules by intravital microscopy. Endothelial expression of intercellular adhesion molecule (ICAM)-1 was measured by the double-radiolabeled monoclonal antibody technique, and β2-integrin expression was measured by flow cytometry. Ischemia-reperfusion elicited significantly larger increases in leukocyte adhesion and emigration in diabetic rats that were prevented by a platelet-activating factor (PAF) receptor antagonist or a leukotriene synthesis inhibitor. Leukotriene B4 (LTB4) superfusion induced similar leukocyte recruitment in diabetic and control rats, whereas PAF elicited larger increases in diabetic rats. CD11a, but not CD11b, expression was higher in leukocytes from diabetic animals. Endothelial ICAM-1 in mesentery and in intestine did not differ between diabetic and control rats. These results indicate that diabetes is associated with an enhanced response to ischemia-reperfusion that depends on both PAF and leukotrienes. An increased sensitivity to PAF, along with an increased CD11a expression, may account for the exaggerated inflammatory response to ischemia-reperfusion in diabetes.

Leukocyte-endothelial cell interaction; platelet-activating factor; leukotrienes; intercellular adhesion molecule-1; integrins

Neutrophils are critically involved with ischemia-reperfusion injury. Strong evidence indicates that these leukocytes contribute to reperfusion injury in a variety of organs including the stomach, intestine, liver, heart, and skeletal muscle (12). This concept is based on experiments showing that neutrophil depletion (14, 31) and inhibition of neutrophil-endothelial cell interactions (19) afford protection from injury after ischemia and reperfusion. Recent evidence indicates that neutrophil infiltration and associated microvascular dysfunction in response to ischemia and reperfusion are exacerbated in the diabetic state (27). Although the adhesion molecules involved in leukocyte recruitment during reperfusion in diabetes have been identified, the physiological mechanisms leading to increased reperfusion-induced leukocyte recruitment in the setting of diabetes remain unknown.

The development of an inflammatory response involves sequential leukocyte-endothelial cell interactions that have been described as rolling, activation, firm adhesion, and emigration (1). Rolling greatly slows the transit of neutrophils through venules, allowing sampling of the local environment or the endothelial cell surface for activating signals. Activated neutrophils upregulate expression of β2-integrins on their surface, and these integrins firmly bind to glycoproteins of the immunoglobulin superfamily, such as intercellular adhesion molecule (ICAM)-1 and ICAM-2, which are expressed on vascular endothelium (11). Therefore, possible factors responsible for the exaggerated inflammatory response to reperfusion in the diabetic state may include an increased sensitivity to the action of proinflammatory mediators generated at reperfusion and/or increased expression of leukocyte and endothelial adhesion molecules.

Several chemical mediators have been implicated in granulocyte accumulation elicited by splanchnic ischemia and reperfusion, the most relevant being platelet-activating factor (PAF) and leukotriene B4 (LTB4). There are two major lines of evidence that implicate PAF and LTB4 as major mediators of reperfusion-induced granulocyte infiltration in the postischemic intestine: 1) levels of both substances rise during the reperfusion period (18, 25, 38); and 2) treatment with PAF- or LTB4-receptor antagonists attenuates reperfusion-induced leukocyte recruitment in the mesentery (18, 20, 38). Therefore, an increased sensitivity to the proinflammatory action of these mediators could account for the exaggerated leukocyte recruitment in diabetes.

Leukocyte adhesion and emigration in response to ischemia-reperfusion has been shown to be critically dependent on interactions between leukocyte β2-integrins and ICAM-1 both in diabetic (27) and in nondiabetic animal models (19), because treatment with blocking monoclonal antibodies against CD18 or ICAM-1 markedly decreases leukocyte adhesion and emigration at reperfusion. It was shown that increased numbers of neutrophils are activated in human diabetic subjects (36), and activation of neutrophils is usually associated with increased β2-integrin expression (33). In addition, an elevated expression of ICAM-1 was demonstrated in choroidal and retinal blood vessels of diabetic patients (24). However, no information in the literature directly addresses whether experimen-
Ischemia-reperfusion damage in diabetes affects the levels of leukocyte integrins or endothelial ICAM-1 expression or whether elevated expression of these adhesion molecules may be a contributory factor to enhanced leukocyte recruitment in the diabetic state.

Thus the current study has three main objectives: 1) to determine whether there is an altered sensitivity to the proinflammatory action of lipid mediators in diabetes; 2) to define the specific contributions of PAF and LTB4 on ischemia-reperfusion-induced leukocyte adhesion and emigration in the diabetic and nondiabetic state; and 3) to assess whether an altered expression of endothelial ICAM-1 or leukocyte integrins represents a contributing factor to the increased inflammatory response to ischemia-reperfusion in diabetes.

**METHODS**

**Animal Model of Diabetes**

Male Sprague-Dawley rats (170–300 g) were obtained from Charles River (Saint Aubin les-Elbeuf, France). After a 7-day adaptation period, diabetes mellitus was induced by injection of streptozotocin (75 mg/kg ip; Zanosar, Upjohn Laboratories, Kalamazoo, MI) diluted in 2 ml of saline. Two days after injection, induction of the diabetic condition (glucose concentration >200 mg/dl) was confirmed by measurement of glucose concentration in blood samples obtained from the tail vein. Rats rendered diabetic were studied 3 wk after streptozotocin injection. This model of diabetes was previously shown to be associated with significant microvascular dysfunction in rats (27). The Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86–23, revised 1985) and the guidelines of procedures for animal experiments from the Generalitat de Catalunya were followed.

**Intravital Microscopy**

Surgical techniques. Animals were anesthetized with an intraperitoneal dose (100 mg/kg body wt in nondiabetic rats and 80 mg/kg in diabetic rats) of thiobutabarbital (Inactin, Research Biochemicals International, Natick, MA). A tracheostomy was performed to facilitate breathing, and the right carotid artery was cannulated to monitor blood pressure throughout the experiment. A midline abdominal incision was made. Vessel loops silicone tubing (Maxxim Medical) was placed around the superior mesenteric artery and exteriorized through the right lateral abdominal wall to induce ischemia by application of traction when indicated.

Microscopy. A section of the mesentery from the small bowel was exteriorized through the midline abdominal incision previously performed. The rats were placed over a steel microscope board in a position that allowed the section of the mesentery to be observed through a glass slide covering a 3.5 × 3.5-cm hole centered on the board. The exposed intestine was covered with a bicarbonate-buffered saline (BBS, pH 7.4)-soaked gauze, and the mesentery was continuously superfused with BBS (37°C) at 2.5 ml/min to avoid dehydration. The superfusate temperature was maintained by pumping the solution through a heat exchanger warmed with a constant-temperature circulator (Haake, Berlin, Germany). The steel board was placed on the stage of an inverted microscope (Diaphot 300, Nikon, Tokyo, Japan) equipped with a CF Fluor ×40 objective lens (Nikon). The preparation was transilluminated with a 12-V, 100-W, direct current-stabilized light source. A charge-coupled device camera (model DXC-930P, Sony, Tokyo) mounted on the microscope projected the image onto a color monitor (Triniton KX-14CP1, Sony), and the images were captured on videotape (SR-5368E videocassette recorder, JVC, Tokyo). Time and date were superimposed on both taped and live images with a date-time generator (KPM Systems, Barcelona, Spain). Rectal temperature was maintained between 36.5 and 37.5°C using an infrared heat lamp and was monitored through a digital thermometer. Single nonbranched venules with diameters ranging between 25 and 35 µm and lengths of >100 µm were studied. Venular diameter (Dv) was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). The number of adherent, emigrated, and rolling leukocytes was measured off-line during playback of videotaped images. A leukocyte was considered adherent to the venular wall if it remained stationary for ≥30 s. Adherent leukocytes were quantified as the number per 100-µm length of venule. Leukocyte emigration was expressed as the number of leukocytes per microscopic field (1.7 × 10 mm²). Rolling leukocytes were defined as those white blood cells circulating at a slower velocity than erythrocytes in the same vessel. Leukocyte rolling velocity was determined from the time required for a leukocyte to traverse a 50-µm distance along the length of the venule and is expressed in micrometers per second. The flux of rolling leukocytes was measured as the number of white blood cells that could be seen rolling within a small (10 µm) viewing area of the vessel, using the same area throughout the experiment. The number of rolling leukocytes per 100-µm venule length was calculated by dividing the leukocyte flux by the leukocyte rolling velocity.

Centerline red blood cell velocity was measured with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University). Venular blood flow was calculated from the product of mean red blood cell velocity (Vmean = centerline red blood cell velocity/1.6) and microvascular cross-sectional area, assuming cylindrical geometry of the vessel. Venular wall shear rate (γ) was calculated from the Newtonian definition $\gamma = 8 \cdot \frac{V_{\text{mean}}}{D_v}$.

**EXPERIMENTAL PROTOCOLS**

**Study 1. Ischemia-Reperfusion: Effect of PAF and LTB4 Blockade**

Once a section of the mesentery had been placed on the microscope board, a single nonbranched venule was selected for study. After a stabilization period of 30 min, images from the mesenteric preparation were recorded on videotape for 5 min. Thereafter, ischemia was induced by pulling the silicone vessel loops tubing, and it was maintained for 10 min; the tube was then removed, allowing blood to reocclude. In all animals studied a decrease in centerline red blood cell velocity of >90% was induced during the period of ischemia. All parameters were measured again 30 min after reperfusion. In some experiments, animals received a dose of WEB-2086 (10 mg/kg body wt), a PAF-receptor antagonist, through the cannulated carotid artery 10 min before induction of ischemia. WEB-2086 was a kind gift from Drs. K. Thomae and A. Zimmer at Boehringer Ingelheim (Ingelheim, Germany). The dose of PAF antagonist used in our experiments was previously shown to be effective in a rat model of ischemia-reperfusion (20). Other groups of animals received a leukotriene synthesis inhibitor, either MK-886 administered orally (10 mg/kg) 10 h before the study or Zileuton (A-64077; 10 mg/kg orally) 6 h before the study. The doses of MK-886 and Zileuton used in our study effectively block LTB4 release in rats (4, 30). MK-886 was a kind donation from Merck.
Frost, and Zileuton was a kind donation from Abbott Laboratories (Madrid, Spain).

Study 2. Sensitivity to Inflammatory Mediators PAF and LTB4

In the experiments designed to evaluate the inflammatory response of the mesenteric venules to PAF or LTB4, images from the mesenteric preparation under baseline conditions were recorded on videotape for 5 min. Afterwards, the exposed tissue was superfused for 30 min with either PAF (100 nM; Sigma Chemical, St. Louis, MO) or LTB4 (20 nM; Calbiochem, San Diego, CA) diluted in BBS. Thereafter, images from the selected venule were recorded for 5 min.

Study 3. Adhesion Molecule Expression

Quantification of endothelial ICAM-1 expression, monoclonal antibodies (MAb) used for the in vivo assessment of ICAM-1 expression were 1A29, a mouse IgG1 against rat ICAM-1 (17), P-23, a nonbinding murine IgG1 directed against human P-selectin (18), and 9B9, a mouse IgG1 directed against human angiotensin-converting enzyme (ACE) that cross-reacts with rat and monkey ACE (6). 1A29 and P-23 were scaled up and purified by protein A/G chromatography at Pharmacia and Upjohn Laboratories (Kalamazoo, MI). Dr. Sergei M. Danilov (Dept. of Pharmacology, University of Illinois, Chicago, IL) generously provided 9B9.

Radioiodination of Monoclonal Antibodies. Binding MAb directed against ICAM-1 (1A29) or ACE (9B9) were labeled with 125I, whereas the nonbinding MAb (P-23) was labeled with 131I. Radioiodination of the MAb was performed by the iodogen method (9). Briefly, 250 µg of protein were incubated with 250 µCi of Na125I or Na131I (Amersham Iberica, Madrid, Spain) and 125 µg of iodogen at 4°C for 12 min. After the radioiodination procedure, the radiolabeled MAb were separated from free 125I or 131I by gel filtration on a Sephadex PD-10 column (Pharmacia LKB, Uppsala, Sweden). Absence of free 125I or 131I was ensured by extensive dialysis of the protein-containing fraction. Less than 1% of the activity of the protein fraction was recovered from the dialysis fluid. Labeled MAb were stored in 500-ml aliquots at 4°C and used within 3 wk after the labeling procedure. The specific activity of labeled MAb was ~0.5 mCi/µg.

Animal Procedures. At the time of the study, anesthesia was induced by intraperitoneal injection of thiobutabarbital (Inactin) at a dose of 100 mg/kg body wt. A tracheostomy was performed on each rat to facilitate breathing throughout the experiment, and the right carotid artery and right jugular vein were cannulated. To measure ICAM-1 expression, a mixture of 5 µg of 125I-labeled ICAM-1 MAb (1A29), 5 µg of 131I-labeled nonbinding MAb (P-23), and 100 µg of nonlabeled ICAM-1 MAb was administered through the jugular vein catheter. This dose of ICAM-1 MAb was shown to saturate all the relevant adhesion receptors expressed on vascular endothelial cells (28). In those experiments in which the relative endothelial surface area was quantified using 9B9, a mixture of 5 µg of 125I-labeled 9B9, 5 µg of 131I-labeled P-23, and 25 µg of nonlabeled 9B9 was administered through the jugular vein catheter. Blood samples were obtained through the carotid artery catheter 5 min after injection of the MAb mixture. Thereafter, the animals were heparinized (1 mg/kg sodium heparin iv) and rapidly exsanguinated by vascular perfusion of sodium bicarbonate buffer via the jugular vein with simultaneous blood withdrawal via the carotid artery. This was followed by perfusion of sodium bicarbonate buffer via the carotid artery after the inferior vena cava was severed at the thoracic level. The mesentery was then harvested and weighed.

Calculations. 125I (binding MAb) and 131I (nonbinding MAb) activities in mesentery and 100-µl aliquots of cell-free plasma were counted in a 14800 Wizard 3 gamma counter (Wallac, Turku, Finland), with automatic correction for background activity and spillover. The injected activity in each experiment was calculated by counting a 5-µl sample of the mixture containing the radiolabeled MAb. The accumulated activity of each MAb in an organ was expressed as the percentage of the injected dose (%ID) per gram of tissue. The formula used to calculate ICAM-1 expression was as follows: ICAM-1 expression = (%ID/g for 125I) - (%ID/g for 131I) × (%ID 125I in plasma)/(%ID 131I in plasma). An identical procedure was used to estimate the relative endothelial surface area of the organs on the basis of accumulation of 9B9.

Quantification of integrin expression on leukocytes. Reagents. RPMI, FCS, and rabbit serum were purchased from Life Technologies (Merelbeke, Belgium). Dextran, polbor 12-myristate 13-acetate (PMA), sodium azide, NaCl, Na2HPO4, KH2PO4, and KCl were all obtained from Sigma Chemicals. Goat anti-mouse IgG (H-L)-fluorescein isothiocyanate (FITC)-labeled antibody was purchased from Caltag Laboratories (Burlingame, CA) and mouse anti-rat CD11a and CD11b from LabGen (Kidlington, UK). Lysing solution was obtained from Becton-Dickinson (San jose, CA).

Flow Cytometry Analysis Studies. Flow cytometry was used to quantify surface expression of CD11a and CD11b on rat leukocytes. Heparinized blood (4 ml) was obtained by heart puncture from diabetic and control rats. Sedimentation of red blood cells was induced by dextran. The leukocyte-rich supernatant was centrifuged and the pellet was suspended in 1 ml of RPMI medium containing 2% FCS and 1% rabbit serum. Aliquots (50 µl) from this mixture were placed on polypropylene tubes and kept at 4°C. PAF, LTB4, and PMA solutions were freshly prepared in RPMI medium with 2% FCS and 1% rabbit serum and were added to the 50-µl blood aliquots to a final concentration of 100 nM PAF, 100 nM LTB4, and 81 nM PMA. Activation of leukocytes was performed by incubation at 37°C with one of these solutions. As negative controls, cells were incubated with medium alone. Incubation with proinflammatory agents or medium was terminated after 30 min by dilution with 3 ml of PBS (in mM: 58.44 NaCl, 101 Na2HPO4, 136.1 KH2PO4, and 27 KCl) with 2% FCS and 0.1% sodium azide at 4°C and centrifugation for 4 min at 400 g and 4°C.

To quantify the expression of adhesion molecules on the surface of leukocytes, 100 µl of a solution containing 20 µg/ml of anti-CD11a or anti-CD11b (final concentration 10 µg/ml) were added to each test tube, and the samples were incubated at 4°C for 45 min. Samples were then washed with 3 ml of PBS with 2% FCS and 0.1% sodium azide at 4°C and centrifuged at 400 g and 4°C for 5 min. Fluorescent labeling was obtained by incubating the samples with a FITC-conjugated goat anti-mouse immunoglobulin (7 µg/ml final concentration) for 45 min at 4°C. The samples were then washed with 3 ml of PBS with 2% FCS and 0.1% sodium azide at 4°C and centrifuged at 400 g and 4°C for 5 min. The red blood cells were then lysed by addition of 2 ml of 1× FACS Brand Lysing Solution to each sample; after gentle vortexing samples were incubated for 10 min at room temperature. After centrifugation for 5 min at 300 g and room temperature, pellets were washed twice with 3 ml of PBS containing 2% FCS and 0.1% sodium azide and centrifuged for 5 min at 200 g and room temperature. Supernatants were then aspirated,
and pellets were resuspended in 300 µl of the same buffer and immediately analyzed.

A FACScan flow cytometer (Becton-Dickinson) and CELLQUEST software were used for acquisition and analysis of the data. The instrument threshold on the forward light-scattering channel was set to exclude platelets. Fluorescence data were acquired in a logarithmic form, the calculation mode was arithmetic, and the results were obtained as linear values. The different populations of leukocytes were identified from forward and side scatter characteristics on dot-plot profiles and were analyzed for fluorescence intensity by using defined gates. A total number of at least 5,000 neutrophils per sample was acquired. Controls included cells stained with the secondary reagent alone or cells stained with an irrelevant isotype-matched control MAb.

Statistical Analysis

All data were analyzed using analysis of variance with Bonferroni as a post hoc test and Student’s paired or unpaired t-test where appropriate. All values are reported as means ± SE. Statistical significance was set at P < 0.05.

RESULTS

Inflammatory Response to Ischemia-Reperfusion: Influence of Diabetes and Role of Inflammatory Mediators

Under baseline conditions diabetic animals had lower blood pressure than controls (controls 132 ± 3.5 vs. diabetic 117 ± 4.7 mmHg, P < 0.01); blood pressure was not significantly modified by ischemia-reperfusion in either group of animals. The diameters of the venules studied were similar in control and diabetic rats (30 ± 1.9 vs. 32 ± 1.5 µm) and were not modified by ischemia-reperfusion. Compared with controls, diabetic rats had a significantly lower basal red blood cell velocity and shear rate in mesenteric venules (Table 1). These parameters were significantly reduced after ischemia-reperfusion in both groups of animals by a similar extent (Table 1).

In basal conditions diabetic rats had significantly higher flux of rolling leukocytes (controls 20.4 ± 4.5, diabetics 83.2 ± 23.7 cells/min, P < 0.05) and number of rolling leukocytes (1.05 ± 0.26 vs. 3.48 ± 0.7 cells/100 µm, P < 0.05), with similar leukocyte rolling velocity [40.5 ± 6.2 vs. 42.7 ± 9.7 µm/s, P = not significant (NS)]. The number of rolling leukocytes was only significantly increased (P < 0.05) compared with basal values early (5–10 min) after reperfusion (controls 2.23 ± 0.98, diabetics 3.97 ± 0.68 cells/100 µm) and returned to baseline values in both groups of animals 30 min after reperfusion (Table 1). Leukocyte adherence and emigration were not different between control and diabetic rats during the control period. Ten minutes of ischemia followed by thirty minutes of reperfusion induced a significant increase in the number of adherent leukocytes in both control and diabetic rats, although in the latter group the number of adherent and emigrated cells was significantly higher than in controls (Fig. 1).

The effects of administration of different inflammatory mediator antagonists on ischemia-reperfusion-induced microvascular alterations are summarized in Table 1. Pretreatment with the PAF-receptor antagonist WEB-2086 did not alter red blood cell velocity or shear rate in baseline conditions but significantly decreased the number of rolling leukocytes in diabetic animals, without affecting basal leukocyte rolling in controls. Treatment with WEB-2086 did not modify the decrease in red blood cell velocity and shear rate observed at reperfusion, and it did not significantly affect the number of rolling leukocytes in control or diabetic rats 30 min after reperfusion, although in the latter group this number tended to be lower in WEB-2086-treated animals than in nontreated diabetic rats (Table 1). Blockade of PAF receptor abrogated ischemia-reperfusion-induced leukocyte adhesion and emigration in control and diabetic animals to the same extent (Fig. 1).

Pretreatment with either of two lipoxygenase inhibitors (MK-886 or Zileuton) had no effect on baseline red blood cell velocity or shear rate in nondiabetic rats. In contrast, lipoxygenase blockade significantly increased baseline red blood cell velocity and shear rate in diabetic animals, up to levels close to those of control rats. The increase in dispersal forces was associated with a significant decrease in the number of rolling leukocytes (Table 1).

Treatment with MK-886 or Zileuton did not prevent the decrease in red blood cell velocity and shear rate

<p>| Table 1. Effects of PAF and LTβ4 antagonists on ischemia-reperfusion-induced venular hemodynamic alterations |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                  | Nontreated      | WEB-2086        | MK-886          | Zileuton        |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th><strong>Treatment Group</strong></th>
<th>Control</th>
<th>Diabetes</th>
<th>Control</th>
<th>Diabetes</th>
<th>Control</th>
<th>Diabetes</th>
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</thead>
<tbody>
<tr>
<td><strong>RBC velocity</strong></td>
<td>3.4 ± 0.3</td>
<td>2.1 ± 0.3a</td>
<td>3.8 ± 0.5</td>
<td>2.2 ± 0.16a</td>
<td>3.5 ± 0.5</td>
<td>2.5 ± 0.3</td>
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<tr>
<td><strong>Shear rate</strong></td>
<td>582 ± 87.3</td>
<td>333 ± 41*</td>
<td>606 ± 67</td>
<td>377 ± 35*</td>
<td>569 ± 84</td>
<td>405 ± 29</td>
</tr>
<tr>
<td><strong>No. of rolling leukocytes</strong></td>
<td>1.05 ± 0.26</td>
<td>3.48 ± 0.7a</td>
<td>1.77 ± 0.7</td>
<td>1.11 ± 0.29*</td>
<td>1.45 ± 0.7</td>
<td>1.0 ± 15*</td>
</tr>
<tr>
<td><strong>Reperfusion</strong></td>
<td>1.8 ± 0.6†</td>
<td>1.06 ± 0.2†</td>
<td>3 ± 0.6†</td>
<td>0.9 ± 0.2†</td>
<td>2.7 ± 0.4</td>
<td>1.6 ± 0.3†</td>
</tr>
<tr>
<td><strong>RBC velocity</strong></td>
<td>289 ± 82†</td>
<td>188 ± 56†</td>
<td>452 ± 76</td>
<td>155 ± 42††</td>
<td>447 ± 54</td>
<td>260 ± 47††</td>
</tr>
<tr>
<td><strong>Shear rate</strong></td>
<td>0.87 ± 0.2</td>
<td>3.26 ± 0.8†</td>
<td>0.59 ± 0.15</td>
<td>1.56 ± 0.46</td>
<td>0.56 ± 0.17</td>
<td>0.68 ± 0.24†</td>
</tr>
<tr>
<td><strong>No. of rolling leukocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.6 ± 0.35</td>
<td>1.8 ± 0.42</td>
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Values are means ± SE of red blood cell (RBC) velocity (in mm/s), shear rate (in s⁻¹), and no. of rolling leukocytes (per 100-µm venule length); n = 5–6 animals/group. PAF, platelet-activating factor; LTβ4, leukotriene B4. *P < 0.05 vs. control rats; †P < 0.05 vs. baseline conditions; ‡P < 0.05 vs. nontreated.
normally observed during reperfusion (Table 1). However, treatment with either drug abrogated leukocyte adhesion and emigration during reperfusion both in control and in diabetic rats (Fig. 1).

Diabetes and Inflammatory Response to Lipid Mediators

Baseline measurements confirmed the alterations in venular microcirculation parameters of diabetic rats described in study 1. Although the diameters of the venules studied were similar in control and diabetic rats (33 ± 1.4 vs. 30 ± 1.1 µm), compared with control rats diabetic animals exhibited significantly lower shear rates (602 ± 44 vs. 352 ± 17 s⁻¹, \( P < 0.0001 \)) and a significant reduction in red blood cell velocity (Table 2). Again, under baseline conditions diabetic rats had significantly higher numbers of leukocytes rolling within the venules (0.4 ± 0.08 vs. 2.1 ± 0.4 cells/100 µm, \( P < 0.005 \)) and a higher rolling flux (11.7 ± 2.2 vs. 51.5 ± 1.4 cells/min, \( P < 0.005 \)) but similar low numbers of adherent and emigrated leukocytes compared with controls (Fig. 1).

Superfusion of the mesentery with either PAF or LTB₄ induced a significant increase in adherent and emigrated leukocytes in all venules studied. However, leukocyte adhesion and emigration in response to superfusion with PAF was remarkably higher in mesenteric venules from diabetic animals than in controls (Fig. 2). In contrast, leukocyte recruitment in response to superfusion with LTB₄ was very similar in diabetic and nondiabetic animals (Fig. 3). Similarly, the number of rolling leukocytes in response to superfusion with PAF was significantly higher in diabetic vessels, whereas in response to LTB₄ diabetic and control rats had a similar number of rolling leukocytes (Table 2). The exacerbated response of diabetic rats to PAF treatment could not be attributable to differences in shear rate, because diabetic and control rats exhibited similar microhemodynamic parameters during superfusion with PAF (Table 2).

Diabetes and Expression of Adhesion Molecules

Neutrophil integrin expression. Under baseline conditions, CD11a expression on neutrophils, monocytes, and lymphocytes of diabetic rats was significantly higher than in cells from control rats (Table 3). CD11a expression did not change significantly after stimulation with PAF, LTB₄, or PMA in either control or diabetic rats (Table 3).

As shown in Table 4, there was no difference in CD11b expression on neutrophils from diabetic and nondiabetic rats in nonstimulated conditions. Neutrophils from both groups of animals showed significantly increased CD11b expression after exposure to PAF or PMA and, in the case of diabetic animals, also in response to LTB₄. The levels of CD11b expression after challenge with these inflammatory mediators were similar in diabetic and control rats.

Table 2. Effect of 30-min PAF or LTB₄ superfusion on hemodynamic parameters from venules of control and diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Baseline Conditions</th>
<th>PAF</th>
<th>LTB₄</th>
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<tr>
<td></td>
<td>Control</td>
<td>Diabetes</td>
<td>Control</td>
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<tr>
<td>Diameter (µm)</td>
<td>30 ± 1</td>
<td>33 ± 1.4</td>
<td>28.2 ± 1.7</td>
</tr>
<tr>
<td>RBC velocity (mm/s)</td>
<td>3.6 ± 0.3</td>
<td>2.3 ± 0.1*</td>
<td>1.8 ± 0.2†</td>
</tr>
<tr>
<td>Shear rate (s⁻¹)</td>
<td>602 ± 45</td>
<td>352 ± 17*</td>
<td>321 ± 67†</td>
</tr>
<tr>
<td>No. of rolling leukocytes</td>
<td>0.45 ± 0.11</td>
<td>2.10 ± 0.85*</td>
<td>0.79 ± 0.32†</td>
</tr>
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</table>

Values are means ± SE of venular diameter (in µm), RBC velocity (in mm/s), shear rate (in s⁻¹) and no. of rolling leukocytes/100-µm venule length; \( n = 6 \) animals/group. *\( P < 0.05 \) vs. control rats; †\( P < 0.05 \) vs. basal measurements (Table 1).
Endothelial ICAM-1 expression. Binding of the anti-ICAM-1 MAb 1A29 (expressed as %ID/g tissue) in the mesentery of diabetic rats was markedly increased compared with control animals (0.118 ± 0.011 vs. 0.228 ± 0.021; P < 0.01). Because diabetic and control rats show remarkable differences in the vascularization of the mesenteric area, with a proliferation of small blood vessels in the mesentery of diabetic rats compared with control rats (27), we estimated endothelial surface area by measuring the accumulation of the anti-ACE MAb 9B9. 9B9 binding (%ID/g tissue) in the mesentery of diabetic rats doubled the binding of control rats (0.078 ± 0.007 vs. 0.139 ± 0.016; P < 0.01), indicating a doubling in endothelial surface area. When accumulation of anti-ICAM-1 MAb was corrected for endothelial surface area, by dividing by mean accumulation of 9B9 in the respective diabetic or control group, no significant differences were observed between diabetic and control animals (1.506 ± 0.142 vs. 1.640 ± 0.153; P = NS).

DISCUSSION

An enhanced inflammatory response to ischemia-reperfusion may render diabetic rats more susceptible to ischemic organ damage (27). In the present study we assessed the potential mechanisms leading to the increased inflammatory response in diabetes. We observed that mesenteric venules of diabetic animals had an increased baseline level of leukocyte rolling compared with controls, which is most likely caused by a reduction in venular shear rate, because the magnitude of increase in the number of rolling leukocytes corresponds to expected values for shear rate-dependent recruitment of rolling leukocytes documented in previous studies (2, 29). In addition, we provide evidence that the increased leukocyte rolling in diabetic rats is dependent on the action of PAF, because treatment with a PAF-receptor antagonist significantly reduced baseline rolling in diabetic animals to levels close to those of control rats without affecting shear rate. Previous studies in nondiabetic animals demonstrated that shear rate-dependent leukocyte rolling is mediated by LTB₄ (2). Results of the present study also show that in diabetic animals blockade of leukotriene synthesis is followed by a reduction in leukocyte rolling. This

Table 3. Effect of PAF, LTB₄, and PMA on CD11a expression on leukocytes of control and diabetic rats

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<tr>
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<th>Neutrophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetes</td>
<td>Control</td>
</tr>
<tr>
<td>Basal</td>
<td>97 ± 5</td>
<td>142 ± 7*</td>
<td>142 ± 8</td>
</tr>
<tr>
<td>PAF</td>
<td>96 ± 7</td>
<td>137 ± 11*</td>
<td>127 ± 6</td>
</tr>
<tr>
<td>LTB₄</td>
<td>113 ± 4</td>
<td>132 ± 12*</td>
<td>164 ± 12</td>
</tr>
<tr>
<td>PMA</td>
<td>80 ± 2</td>
<td>107 ± 10*</td>
<td>159 ± 9</td>
</tr>
</tbody>
</table>

Values, expressed as fluorescence intensity (in arbitrary units), are means ± SE; n = 6 animals/group. PMA, phorbol myristate acetate. *P < 0.05 vs. control rats; †P < 0.05 vs. baseline conditions.
are in keeping with previous evidence demonstrating leukocyte recruitment after reperfusion. These results synthesis inhibitors (MK-886 or A-64077), abrogated antagonist WEB-2086, or either of two leukotriene reperfusion, because treatment with the PAF-receptor to mount the inflammatory response to ischemia-diabetic rats and diabetic rats compared with controls. One of the mechanisms that may increase the sensitivity to PAF in diabetic animals is the decrease in PAF-acetylhydrolase activity that has been documented in plasma of streptozotocin-induced diabetic rats (35). Taken together, these observations indicate that an increased sensitivity to the proinflammatory action of PAF, but not to LTB₄, is probably the basis for the exaggerated inflammatory response to ischemia-reperfusion in the diabetic state.

Another potential mechanism that might contribute to enhance the inflammatory response in diabetes is activation of adhesion molecules involved in leukocyte recruitment at reperfusion. It has been shown that in both nondiabetic and diabetic rat models of ischemia-reperfusion, immunoneutralization of either β₂-integrins or ICAM-1 blunts ischemia-reperfusion-induced leukocyte recruitment (19, 27). A growing body of evidence shows that the function and mechanical properties of granulocytes are altered in diabetes (8, 16, 23), and these cells generate larger quantities of toxic oxygen radicals (10) than granulocytes isolated from nondiabetics. Activation of leukocytes leads to functional activation of integrins constitutively expressed on the surface of the cells (CD11a/CD18) (32, 34) and translocation to the surface membrane of integrin molecules stored in granules (CD11b/CD18) (3, 15). Therefore, increased β₂-integrin activation/expression in the diabetic state might potentially contribute to enhance leukocyte recruitment. The results in this report indicate that nonstimulated leukocytes (neutrophils, monocytes and lymphocytes) from diabetic rats exhibit a higher expression of CD11a/CD18 and that the level of expression of this integrin is not affected by exposure to PAF, LTB₄, or PMA. The increased expression of CD11a/CD18 may contribute to enhance leukocyte adhesiveness in response to stimuli that activate this integrin, including ischemia-reperfusion (13, 37). The lack of change in expression of CD11a/CD18 after stimulation is in keeping with previous observations (5). In contrast with the differences in CD11a/CD18 expression, baseline CD11b/CD18 expression on neutrophils and on monocytes was similar in diabetic and nondiabetic animals. Increments in expression of this integrin after stimulation were of similar magnitude in diabetic and nondiabetic animals. Therefore, changes in expression of CD11b/CD18 do not seem to contribute to enhance leukocyte recruitment in the diabetic state.

Another adhesion molecule that has proved crucial in leukocyte recruitment after ischemia-reperfusion in control and diabetic animals is ICAM-1. Immunoneutralization of this adhesion molecule has a potent blocking effect on leukocyte recruitment at reperfusion (21, 27). On the other hand, increased expression of ICAM-1 has been documented by immunohistochemistry in diabetic human retina and choroid (24) and elevated levels of soluble ICAM-1 have been reported in serum of patients with insulin-dependent diabetes mellitus (22), although the latter finding was not confirmed in a recent study (26). We found that expression of endothelial ICAM-1 in mesenteric venules, when corrected for endothelial surface area, is very similar in control and diabetic animals. We only assessed constitutive expression of ICAM-1, because expression of this adhesion molecule is under transcriptional regulation (7) and the duration of the reperfusion period in our study was only 30 min, too short a period to allow for significant change in ICAM-1 expression in diabetes.

### Table 4. Effect of PAF, LTB₄, and PMA on CD11b expression on neutrophils and monocytes of control and diabetic rats

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>Control</td>
<td>Diabetes</td>
</tr>
<tr>
<td>Basal</td>
<td>33 ± 8</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>PAF</td>
<td>40 ± 8†</td>
<td>40 ± 8†</td>
</tr>
<tr>
<td>LTB₄</td>
<td>38 ± 7</td>
<td>38 ± 4†</td>
</tr>
<tr>
<td>PMA</td>
<td>157 ± 49†</td>
<td>151 ± 25†</td>
</tr>
</tbody>
</table>

Values, expressed as fluorescence intensity (in arbitrary units), are means ± SE; n = 7 animals/group. †P < 0.05 vs. baseline conditions.
response to ischemia-reperfusion. The apparent discrepancy between these findings and previous evidence of increased ICAM-1 expression in ocular vessels (24) may be related to some species or organ specificity of vascular alterations in diabetes. Therefore, in the experimental diabetes model used in the present study, changes in ICAM-1 expression do not account for the increased leukocyte recruitment in mesenteric venules, although we cannot rule out that upregulation of this adhesion molecule contributes to ischemic damage in certain organs in long-standing human diabetes. The possibility exists that an increased expression of other adhesion molecules such as P-selectin or vascular cell adhesion molecule-1 may also contribute to enhance leukocyte recruitment in the diabetic state. In fact, an increase in P-selectin expression has been documented in the diabetic human retina and choroid (24).

In summary, the results of the present study indicate that the diabetic state markedly enhances the inflammatory response to ischemia-reperfusion and that participation of PAF and leukotrienes is essential in the pathogenesis of this response, both in control and diabetic animals. Our results also suggest that an increased sensitivity to the proinflammatory action of PAF, but not to LTB4, may be the basis for the exaggerated inflammatory response to ischemia-reperfusion in the diabetic state. Increased expression of CD11a/CD18 on neutrophils, monocytes, and lymphocytes, which is activated by ischemia-reperfusion (13, 37) or PAF (5), may also contribute to enhance leukocyte adhesion in response to this inflammatory stimulus. These findings are especially relevant for the development of new therapeutic strategies to prevent ischemic damage and indicate that in the diabetic condition, agents that block the action of PAF can be particularly beneficial.

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