Role of platelet-activating factor in ischemia/reperfusion-induced leukocyte adherence

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Kubes, Paul, Geoffrey Ibbotson, Janice Russell, John L. Wallace, and D. Neil Granger. Role of platelet-activating factor in ischemia/reperfusion-induced leukocyte adherence. Am. J. Physiol. 259 (Gastrointest. Liver Physiol. 22): G300-G305, 1990.—The objective of this study was to determine whether platelet-activating factor (PAF) mediates the leukocyte-endothelial cell interactions elicited by ischemia/reperfusion. The rates of adherence and extravasation of leukocytes were monitored in cat mesenteric venules subjected to 60 min of ischemia (blood flow reduced to 20% of control) followed by 60 min of reperfusion. Leukocyte rolling velocity, red blood cell velocity, and vessel diameter were also measured. The experiments were performed in control (untreated) animals and in animals pretreated with one of two PAF receptor antagonists, i.e., BN 52021 or WEB 2086. The responses of venular blood flow, wall shear rate, and vessel diameter did not differ between the three groups. In the control group, 1 h of ischemia was associated with significant adherence and extravasation of leukocytes, with reperfusion greatly enhancing these responses. The rates of leukocyte adherence and extravasation during reperfusion were greatly attenuated by both PAF antagonists. Furthermore, the proportion of adherent leukocytes that ultimately extravasated during reperfusion was markedly reduced by WEB 2086. These results suggest that PAF plays an important role in mediating the adhesive interaction between circulating leukocytes and microvascular endothelium induced by ischemia/reperfusion and that the phospholipid promotes the leukocyte extravasation associated with ischemia/reperfusion.

leukocyte adhesion; leukocyte-endothelial cell interactions; inflammation

Reperfusion of ischemic tissues is associated with a variety of microvascular alterations, including endothelial cell swelling, increased vascular permeability, enhanced capillary filtration, and capillary plugging (4–6, 8). Although much attention has been devoted to the role of leukocytes in mediating the reperfusion-induced microvascular dysfunction, relatively little is known about the mechanisms that are responsible for promoting the adherence and extravasation of leukocytes in postischemic microvessels. It has recently been demonstrated that reperfusion of ischemic mesenteric (7) or muscle venules (23) results in a dramatic increase in the rate of leukocyte adherence and extravasation. The reperfusion-induced increases in leukocyte adherence and extravasation were significantly attenuated by pretreatment with either allopurinol or superoxide dismutase (SOD). These observations are consistent with earlier work which demonstrates that the granulocyte accumulation (assessed by tissue myeloperoxidase measurements) induced by reperfusion of ischemic intestinal mucosa is reduced by allopurinol and SOD (9).

Although the results of the aforementioned studies implicate xanthine oxidase–derived oxidants in reperfusion-induced leukocyte recruitment, the chemical substance that ultimately mediates this process remains undefined. While it appears unlikely that oxygen radicals can directly initiate leukocyte adherence and activation, it is conceivable that oxidants can interact with endothelial cell membranes to activate phospholipase A₂ and consequently lead to the formation of powerful leukocyte chemoattractants such as platelet-activating factor (PAF) and leukotriene B₄. Indeed, there is evidence that oxidants can stimulate the synthesis of PAF by cultured endothelial cells and subsequently promote the adherence of neutrophils to endothelial monolayers (14). This observation, coupled to the fact that local administration of PAF leads to an increase in both permeability and leukocyte adherence in venules (1, 2, 13), lends support to the possibility that PAF mediates reperfusion-induced leukocyte adherence and extravasation. The objectives of this study were to determine whether 1) tissue levels of PAF are altered in intestinal segments subjected to ischemia/reperfusion and 2) PAF antagonists modify the leukocyte adherence and extravasation in mesenteric venules subjected to ischemia/reperfusion.

MATERIALS AND METHODS

Animal preparation. Cats (n = 18) weighing between 2 and 3 kg were initially anesthetized with 50 mg/kg ketamine hydrochloride. The left jugular vein was cannulated, and anesthesia was maintained by administration of pentobarbital sodium. A tracheotomy was performed, and animals were artificially ventilated using a Harvard respirator. The right femoral artery was cannulated to measure systemic arterial pressure. After a midline abdominal incision, the small intestine was isolated from the ligament of Treitz to the ileocecal valve, without disturbing blood and lymph vessels. The remainder of the small and large intestine was extirpated. The animals then received heparin (3,000 U/kg iv), and an arterial circuit was established from the left femoral
artery to the superior mesenteric artery. Intestinal blood flow was measured by a flow probe that was interposed within the arterial circuit. Superior mesenteric arterial pressure was measured via a pressure port on the flow probe. Systemic and superior mesenteric arterial pressures were measured with Statham P23A transducers and continuously recorded with a Grass physiological recorder.

**Intravital microscopy.** After completion of surgery, animals were placed in a right lateral recumbent position on an adjustable Plexiglas microscope stage, and a segment of the midjejunum was exteriorized through the abdominal incision, with great care taken to avoid trauma to the exposed bowel and mesentery. The mesentery was prepared for in vivo microscopic observation according to previously published reports (7, 11). Briefly, the mesentery was draped over an optically clear viewing pedestal that allowed for transillumination of a 3-cm square section. The exposed bowel wall was draped with saline-soaked gauze. The mesentery was covered with Saran Wrap (Dow Corning) and continuously suffused with a warm (37°C) bicarbonate-buffered salt solution (pH = 7.4; Po2 = 40 mmHg) at a rate of 2.0 ml/min. The temperature of the pedestal was maintained at 37°C with a constant-temperature circulator (model 80, Fisher Scientific).

Single unbranched mesenteric venules (25–35 μm diam; 250 μm length) were observed though an intravital video microscope (Leitz, Ortholux II). The mesentery was transilluminated, and a video camera (Dage, MTI) mounted on the microscope projected the image onto a black-and-white monitor. The images were recorded using a video cassette recorder for playback analysis.

Venular diameter (D) was measured using a video caliper, and red blood cell centerline velocity was measured using an optical Doppler velocimeter (Microcirkulation Research Institute, Texas A&M University, College Station, TX). Venular blood flow was calculated as the product of mean red blood cell velocity [V

The number of adherent and extravasated leukocytes and leukocyte rolling velocity were determined off-line during playback of videotaped images. Due to the small diameter of the venules, leukocytes adherent to any surface of the vessel wall could be visualized. A leukocyte was defined as adherent to venular endothelium if it was stationary for 30 s or longer. The rate of leukocyte adherence during reperfusion was expressed as the average number per 100 μm length of venule per minute. The rate of leukocyte extravasation was calculated as the difference between the number of extravasated leukocytes in the field of view at the beginning and end of the reperfusion period. Rolling leukocytes were defined as white blood cells that moved at a velocity less than that of red blood cells in the same vessel. Leukocyte rolling velocity was determined as the time required for a leukocyte to traverse a given length of venule.

**Protocols.** When arterial pressure, intestinal blood flow, and red blood cell velocity were in a steady state, images from the mesenteric preparation were recorded on videotape for 10 min. The superior mesenteric arterial circuit was then partially occluded so that intestinal blood flow fell to 20% of baseline. The bowel was maintained in an ischemic state for 60 min, with video recordings made during the final 10 min of ischemia. The arterial occlusion was then released and video recordings were obtained at 10 and 60 min after reperfusion. In five animals, the control responses to ischemia/reperfusion were obtained. Two additional groups of cats (n = 5 each) were pretreated systemically (iv) with the PAF antagonist BN 52021 (20 mg/kg) or WEB 2086 (10 mg/kg) and then subjected to the above protocol.

**PAF measurements.** In another group of cats (n = 3) jejunal segments were subjected to the ischemia-reperfusion protocol, and a mucosal sample (300 mg) was obtained under control conditions, after 1 h of ischemia, and 10 and 60 min after reperfusion. Ex vivo production of PAF was determined using the procedure previously described in our laboratory (22). Briefly, the samples were added to 1 ml of 0.25% bovine serum albumin (BSA), vortexed, and incubated for 10 min (37°C). The mixture was added to 2 ml cold acetone (−20°C), and after centrifugation (2,000 g for 5 min) the supernatant was added to 2 ml of chloroform and again centrifuged. The PAF in the organic phase was dried under a stream of nitrogen and then characterized by thin layer chromatography (TLC). The dried extracts were dissolved in chloroform-methanol (1:1 vol/vol) and applied to TLC plates, which were developed in chloroform-methanol-water (65:35:6 vol/vol/vol). Zones from the platelet that comigrated on adjacent lanes with authentic [3H]PAF were transferred to 0.25% BSA in saline, vortexed, and reextracted with acetone and chloroform (1:1). Subsequently, the dried organic phase was resuspended in 20 mM tris(hydroxymethyl)aminomethane buffer (pH 8.0) containing 0.25% BSA, and the PAF activity of each TLC extract was bioassayed as the aggregation of rabbit washed platelets.

Rabbit blood was collected in trisodium citrate (0.32% final concentration) and centrifuged to prepare platelet-rich plasma. The platelets were subsequently washed free of plasma with protacyclin (Wellcome Foundation) and resuspended in Tyrode solution as described by Radomski and Moncada (17). The extracted PAF was bioassayed against concentration-aggregation curves generated using authentic PAF (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine, 99% pure, Sigma) and a Seinco dual-channel aggregometer (DP-247R). The pro-aggregatory activity of the extracts was confirmed as PAF, since it was completely inhibited by preincubating the platelet suspensions for 1 min with BN 52021 (1 μm).

The data were analyzed using standard statistical analyses, i.e., one-way analysis of variance and Scheffe’s test and the Mann-Whitney U test for PAF tissue levels. All values are reported as means ± SE, and statistical significance was set at P < 0.05.

**RESULTS**

Figure 1 presents the levels of PAF detected in the intestinal mucosa during the control, ischemic, and re-
Levels of platelet-activating factor (PAF) during control, ischemia, and reperfusion (10 and 60 min). A significant increase in PAF levels was observed after 60 min of reperfusion. PAF levels were not different among the three groups during the control period and did not change after the administration of WEB 2086 or BN 52021.

Table 1 presents the data for 1) the average rate of leukocyte extravasation (RAE) and 2) the rate of leukocyte extravasation (RLE). The RLE of leukocytes adhering to venule walls was 210% larger in the WEB 2086-treated animals compared with the two other groups.

Leukocyte adherence and extravasation was not different among the three groups during the control period, and did not change after the administration of WEB 2086 or BN 52021. The number of adherent leukocytes increased from 4.8 ± 0.5 to 13 ± 2, and the number of extravasated leukocytes was augmented from 11 ± 11 to 21 ± 0.2. Pretreatment with either WEB 2086 or BN 52021 reduced RA by 36-40% (P < 0.05). The ratio of RA to RAE, expressed as the number of leukocytes extruding per minute per 100 μm length of venule, was 0.21 ± 0.04. Pretreatment with WEB 2086 reduced RA by 36-40% (P < 0.05). The ratio of RA to RAE was also effective at lowering RA in one of the experimental groups until after 60 min of reperfusion.

Table 1 summarizes the hemodynamic and leukocyte responses that were observed in the three groups of intravital microscopy experiments. The hemodynamic data, including superior mesenteric arterial pressure, superior mesenteric arterial resistance, venular blood flow, venular wall shear rate, and leukocyte rolling velocity, were not different among the three groups at any time during the experiment. The ratio of leukocyte rolling velocity to erythrocyte velocity (V_leukocyte/V_erythrocyte) did not differ among experimental groups until after 60 min of reperfusion, at which point V_leukocyte/V_erythrocyte was 210% larger in the WEB 2086-treated animals compared with the two other groups.

Leukocyte adherence and extravasation was not different among the three groups during the control period. Pretreatment with either BN 52021 or WEB 2086 reduced RLE by 35-40% (P < 0.05). The two PAF antagonists were also effective at lowering RLE in one of the experimental groups until after 60 min of reperfusion.

The ratio of RLE to RLA, which reflects the proportion of adherent leukocytes that ultimately extravasate during reperfusion, was significantly reduced by WEB 2086 but not by BN 52021.
ponents to form and/or release substances that activate unknown, there is evidence which suggests that lipid peroxidation and phospholipase A2 activation are in-

increased microvascular permeability induced by reper-

fusion are significantly attenuated by xanthine oxidase

thine oxidase, oxygen radicals, and leukocyte adherence

remains undefined. However, it has been suggested that

xanthine oxidase-derived oxidants, produced at the time

of reperfusion, may interact with cell membrane com-

ponents located on the activated leukocyte and hydro-

dynamic forces tending to sweep it away from the endo-

thelial surface. Nonetheless, our results indicate that an

increase in hydrodynamic dispersal forces cannot explain

the attenuated adherence produced by PAF antagonists, since no differences in venular wall shear rate, blood

flow, or leukocyte rolling velocity were noted among the

three groups of animals (Table 1). Furthermore, it ap-

pears unlikely that the PAF antagonists exert a direct
effect on neutrophil adherence, since neither BN 52021 nor WEB 2086 had an effect on base-line (preischemic)

neutrophil adherence values.

A likely explanation for the beneficial effects of PAF

antagonists in our model of reperfusion-induced leuko-
cyte adherence is that these agents are competing with

newly formed PAF for receptor sites on the leukocyte

and/or endothelial cell membranes that modulate leu-
cyte-endothelial cell adhesion. This possibility is sup-

ported by several lines of evidence: 1) reperfusion of the

ischemic intestine is associated with an increased tissue

centration of PAF (Fig. 1), 2) PAF infused into the

superior mesenteric artery promotes leukocyte adherence

to mesenteric venular endothelium (13), and 3) PAF

receptor antagonists attenuate the adhesive interaction

between leukocytes and endothelium that is induced by

exposure of endothelial monolayers to oxidants such as

hydrogen peroxide (14). The latter observation, coupled
to the results of this study, provide some support for the

view that reperfusion-induced oxidant formation may

lead to the production of PAF and consequently promote

the adherence of neutrophils to microvascular endo-
thelium. Nonetheless, it remains unclear whether the PAF

formed in response to ischemia/reperfusion directly re-

sults in the expression of leukocyte adhesion molecules

or indirectly promotes adherence by causing the release
of other proinflammatory agents such as leukotriene B₄ and C₄ (12, 21).

Although the mechanisms involved in the emigration of leukocytes from the blood stream are not fully understood, it is well recognized that neutrophil adherence to microvascular endothelium is an initial and rate-limiting step in this process. Since adherence is a prerequisite for diapedesis, one would predict that the inhibitory effect of PAF antagonists on reperfusion-induced leukocyte adherence should be associated with a corresponding reduction in the rate of leukocyte extravasation. Indeed, the results of our studies (Fig. 2) indicate that WEB 2086 and BN 52021 both attenuate the rate of leukocyte extravasation normally observed after reperfusion of the ischemic mesentery. Of particular interest, however, is our observation that WEB 2086 reduces the proportion of adherent leukocytes that ultimately extravasates during reperfusion (Rₑ₋ₑ/Rₑₐ) by 80% (Fig. 2). This reduction suggests that the PAF antagonist interferes with leukocyte extravasation by a process that is independent of adherence. It remains unclear exactly how WEB 2086 alters the extravasation process and why this effect was not produced by BN 52021. The different responses to BN 52021 and WEB 2086 suggest that either WEB 2086 exerts an influence on leukocyte extravasation that is independent of its actions on PAF receptors or that some antagonists act on different PAF receptor populations on the leukocyte and/or endothelial cell (20). It is also feasible that WEB 2086 is more potent than BN 52021, possibly due to different bioavailability. Since PAF is known to stimulate degranulation of neutrophils (15, 18), it is conceivable that the phospholipid participates in the extravasation process by promoting the limited release of neutrophilic proteases (e.g., elastase) and oxidants, which, in turn, facilitate the migration of phagocytic cells across restrictive barriers such as the basement membrane (10, 19).

It is not clear from our study as to the relative contribution of ischemia and reperfusion to the leukocyte-endothelial cell interactions observed after 1 h of reperfusion. However, in a previous study we observed that the neutrophil accumulation in intestinal mucosa exposed to 3 h of ischemia is not different from the neutrophil accumulation observed after 1 h of ischemia. This observation suggests that the additional increment in neutrophil accumulation observed 10 min after reperfusion can be attributed to events associated with reperfusion per se. A role for reperfusion is also supported by recent observations that leukocyte adherence and extravasation in mesenteric venules increase dramatically within 10 min after reperfusion (24).

In conclusion, the results of this study indicate that PAF plays an important role in the leukocyte adherence and extravasation induced by reperfusion of the ischemic cat mesentery. Our results indicate that PAF is formed in response to ischemia/reperfusion and that the phospholipid subsequently promotes an adhesive interaction between circulating leukocytes and venular endothelium. PAF also appears to promote the extravasation of leukocytes by mechanisms that are both dependent and independent of leukocyte-endothelium adhesion. Additional studies are needed to define the mechanisms responsible for the enhanced production of PAF after reperfusion of ischemic tissues and to determine how the phospholipid modulates the leukocyte adhesion and diapedesis induced by ischemia/reperfusion.

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