Endothelin-1 causes P-selectin-dependent leukocyte rolling and adhesion within rat mesenteric microvessels

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Sanz, Maria-Jesus, Brent Johnston, Andrew Issekutz, and Paul Kubes. Endothelin-1 causes P-selectin-dependent leukocyte rolling and adhesion within rat mesenteric microvessels. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1823–H1830, 1999.—Endothelin-1 (ET-1) is a potent vasoconstrictor postulated to play a role in hypertension, ischemia-reperfusion, and atherosclerosis. In addition to these contributions, it has been also proposed to induce leukocyte-endothelial cell interactions. The aim of the present study was to assess the mechanisms of action of ET-1 on leukocyte recruitment in vivo. Intravital microscopy of the rat mesenteric postcapillary venules was used. Ten minutes after 1 nM ET-1 superfusion, a significant increase in leukocyte rolling (77.5 ± 22.6 vs. 20.5 ± 4.5 cells/min) and adhesion (15.5 ± 2.9 vs. 3.0 ± 0.8 cells/100 μm) but not emigration was observed. These effects were found not to be mediated by mast cell activation. No platelet-endothelial cell interactions were detected in this in vivo system and furthermore, flow cytometry analysis revealed no increase of P-selectin expression in rat platelets on ET-1 stimulation. Pretreatment of animals with an anti-rat P-selectin monoclonal antibody (mAb) dramatically reduced leukocyte rolling and adhesion by 100 and 94%, respectively when compared with control mAb-treated animals. At this dose of ET-1, a very transient decrease in shear rate was detected, arteriolar diameter was significantly reduced but venular diameter remained unchanged. A similar mechanical reduction in blood flow did not induce leukocyte recruitment. Thus this study demonstrates that ET-1 can directly cause significant leukocyte rolling and adhesion adding to its potential pathophysiological role in the development of disease states of the cardiovascular system.

LEUKOCYTES MOVING at very high speeds in the mainstream of blood depend on critical endothelial adhesion molecules to make interactions with the vessel wall before firmly adhering to and migrating out of the vasculature (31). This initial capture of leukocytes, which is also known as tethering and rolling, is mediated by the selectins, including P-selectin and E-selectin and also perhaps vascular adhesion molecule-1 (VCAM-1) (1, 17, 31). Leukocyte capture in the initial stage (minutes) of the inflammatory response is dependent on P-selectin (10) because neither E-selectin nor VCAM-1 is constitutively expressed and both require de novo protein synthesis, which occurs over hours. P-selectin is presynthesized and stored in Weibel-Palade bodies of endothelial cells and is rapidly mobilized to the cell surface after exposure of endothelium to histamine, cysteinyl leukotrienes, and oxidants. In laminar flow chamber studies, perfusion of white cells over histamine-stimulated endothelial monolayers induces tethering and rolling of neutrophils that is entirely dependent on P-selectin (19). Similarly, using intravital microscopy in vivo, investigators have been able to demonstrate that histamine will induce rapid leukocyte rolling (24) and similar results were noted for leukotriene C4 (LTC4) and H2O2 (18, 20). More recently a new class of mediators, the kinins, and specifically bradykinin, have also been shown to induce leukocyte rolling via P-selectin (32). Many of the aforementioned mediators and their precursors are released by mast cells, and activation of mast cells can induce P-selectin-dependent leukocyte rolling and subsequent adhesion (9), suggesting that the mast cell may be a key route of P-selectin-dependent leukocyte recruitment.

Endothelin-1 (ET-1) is a molecule that has numerous biological properties in vitro including mast cell activation (5a, 33, 34). However, ET-1 has generally not been thought of as an important mediator of leukocyte recruitment in vivo but rather as a regulator of blood pressure and as a pathological agent in hypertension. Nevertheless, a few studies have noted some effects on humoral cells; ET-1 induces platelet aggregation and may induce leukocyte adhesion (3, 6, 11, 23). It should, however, be noted that ET-1 can also release nitric oxide (an antiadhesive agent) and has been reported to reduce leukocyte adhesion (30). One potential explanation for the proadhesive effects of ET-1 may be its vasoconstrictive effects which decrease hemodynamic factors that normally push leukocytes within postcapillary venules. The reduction in venular shear can induce leukocyte-endothelium interactions and may even impact on platelet interactions with the endothelium. However, whether ET-1 directly affects leukocyte-endothelium interactions and platelet-endothelium interactions, whether mast cell activation is a key element, and whether the reduction in shear forces can explain most of the adhesion, remain unknown.

With the use of intravital microscopy, we were able to systematically assess leukocyte and platelet behavior in single postcapillary venules, hemodynamic parameters within the vessel, and the state of mast cell activation surrounding the vessel under study. This permitted us to first test the hypothesis that ET-1 increases leukocyte-endothelium and platelet-endothelium interactions. Second, we examined whether reductions in shear forces were responsible for leukocyte-endothelium interactions and whether the increase in...
rolling was a result of P-selectin. Finally, using multiple approaches we examined a role for mast cells in ET-1-induced leukocyte recruitment. First, with the use of a novel online assessment of mast cell activation, we examined whether ET-1 could stimulate mast cells, and second, we determined whether mast cell stabilization could prevent leukocyte recruitment. Our results demonstrate a very rapid increase in leukocyte rolling that was entirely P-selectin dependent. Additionally, a transient increase in leukocyte adhesion was also noted, but the cells did not migrate out of the vasculature. Associated with the increased rolling and adhesion was a brief 2- to 5-min reduction in shear forces within the microvasculature. However, in the absence of ET-1 physical reduction in shear forces for the same duration did not induce any rolling or adhesion in the postcapillary venules, suggesting that altered hemodynamics did not contribute to enhanced cell-to-cell interactions. Finally, a systematic approach failed to reveal a role for mast cells in the ET-1-induced leukocyte recruitment.

METHODS

Intravital microscopic studies. The experimental preparation used in this study is the same as described previously (9). Briefly, rats (~200 g) were fasted for 24 h and anesthetized with pentobarbital sodium. The jugular vein was cannulated, and anesthesia was maintained by the administration of additional anesthetic. Systemic arterial pressure was monitored by a pressure transducer (Statham P23A; Gould, Oxnard, CA) connected to a catheter in the carotid artery. Blood pressure was continuously recorded with a physiological recorder (Grass Instruments, Quincy, MA).

A midline abdominal incision was made, and the rats were placed in a supine position on an adjustable Plexiglas microscope stage. A segment of small intestine was exteriorized through the abdominal incision. The mesentery was draped over an optically clear viewing pedestal that allowed for transillumination of a 3-cm segment of tissue. The temperature of the pedesetal was maintained at 37°C with a constant temperature circulator (model 80; Fisher Scientific, Pittsburroh, PA). The exposed bowel was draped with saline-soaked gauze while the remainder of the mesentery was covered with Saran Wrap (Dow Corning, Midland, MI). The exposed mesentery was suffused with warmed bicarbonate-buffered saline (pH 7.4). The mesenteric preparation was observed through an intravital microscope (Optiphot-2; Nikon, Mississauga, Canada) with a ×25 objective lens (Wetzlar L25/0.35; E. Leitz, Munich, Germany) and a ×10 eyepiece. The image of the microcirculatory bed (×1,400 magnification) was recorded using a video camera (Digital 5100; Panasonic, Osaka, Japan) and a video recorder (NV8950; Panasonic). The temperature of the animal was maintained at 37°C using an infrared heat lamp.

Single unbranched mesenteric venules (25–40 µm diameter, 250 µm length) were selected for each study. Venular diameter was measured either on- or offline using a video caliper (Microcirculation Research Institute, Texas A & M University, College Station, TX). The number of rolling and adherent leukocytes and platelets was determined offline during play-back analysis. Rolling leukocytes were defined as white blood cells that moved at a velocity less than that of erythrocytes in a given vessel. The number of rolling leukocytes (flux) was counted using frame-by-frame analysis. To obtain a complete leukocyte rolling velocity profile, the rolling velocity of all leukocytes entering the vessel was measured. A leukocyte was defined as adherent to venular endothelium if it remained stationary for >30 s. Adherent cells were measured at 15-min intervals as described in the experimental protocol and expressed as the number per 100-µm length of venule. Red blood cell velocity ($V_{\text{RBC}}$) was measured using an optical Doppler velocimeter (Microcirculation Research Institute), and mean red blood cell velocity $V_{\text{mean}}$ was determined as $V_{\text{RBC}}/1.6$ (15). Wall shear rate was calculated on the basis of the Newtonian definition: shear rate = $8 \times (V_{\text{mean}} / D_v )^{-1}$, where $D_v$ is the venular diameter.

Experimental protocol. Experiments were carried out in four groups of animals. In the first group of animals, baseline measurements of blood pressure, $V_{\text{RBC}}$, vessel diameter, leukocyte rolling flux, rolling velocity, adhesion, and emigration were obtained after 30 min of steady state. The mesentery was then superfused with ET-1 at 1 nM in bicarbonate-buffered saline for 2 min, rinsed thoroughly after this time period, and all parameters were measured again at 10 and 30 min after ET-1 superfusion. Preliminary work revealed that 10 nM ET-1 permanently reduced blood flow and 0.1 nM had less profound and less consistent results than 1 nM. In the next two groups of animals, rats received a bolus injection of a monoclonal antibody directed against rat P-selectin (RMP-1, IgG2a) or the nonblocking anti-rat P-selectin monoclonal antibody (mAb) (RP-2, IgG1) 5 min before ET-1 superfusion, both mAbs were used at 2.5 mg/kg as previously described (16).

To confirm that ET-1-induced responses within the rat mesenteric microcirculation were not caused by a blood flow reduction another set of experiments were performed where blood flow was mechanically reduced for 10 min using a pressure cuff around the mesenteric artery. To mimic the transient effects of ET-1 the cuff was loosened after 5–10 min.

Finally, the degree of mast cell activation induced by ET-1 was determined as previously described (9). Briefly, 0.001% ruthenium red was added to ET-1 superfusion buffer throughout the experiment. After 40 min of ET-1 superfusion compound 48/80 (CMP 48/80) at 1 µg/ml was added to the superfusion buffer to have a positive control of mast cell activation. With the use of a video capture board (Visionplus AT-OFG, Imaging Technology, Bedford, MA) and a computer-assisted digital imaging processor (Kontron, Bioscan, Edmonds, WA) all color images were converted to digitized gray scales and phase inverted. The relative light intensity of individual mast cells was measured, and data are presented as the degree of stain (intensity) relative to background. The mast cells were gated, and the degree of stain uptake was assessed by the computer system to eliminate any variability between individuals and to avoid any potential bias.

Determination of surface expression of P-selectin in platelets. The expression of P-selectin was determined in platelets from the peripheral blood of rats. Citrated whole blood was diluted 1:10 in modified Tyrode buffer (137 mM NaCl, 2.8 mM KCl, 1 mM MgCl₂, 12 mM NaHCO₃, 0.4 mM Na₂HPO₄, 0.35% BSA, 10 mM HEPES, 5.5 mM glucose, pH 7.4). Diluted whole blood (25 µl) was then mixed with 5 µl of primary anti-rat P-selectin mAb (RP-2, mouse IgG1) or an isotype-matched control mAb (mouse IgG1) followed immediately by 5 µl of either Tyrode buffer, ADP, or ET-1. Final concentrations of the agonists were 80 µM for ADP and 1 nM and 100 nM for ET-1. Samples were kept at room temperature for 15 min and then were incubated with saturating amounts (5 µl) of goat anti-mouse IgG1 fluorescein isothiocyanate (FITC)-conjugated mAb (Fc specific) in the dark for another 15 min.

Flow cytometry. All the analyses were performed with an EPICS XL-MCL Flow Cytometer (Coulter Electronics, Hi-
aleah, FL) with a 15-mW argon laser tuned at 488 nm. The instrument was set to measure forward-angle light scatter (FS), side-angle light scatter (SS) and FITC fluorescence (FL1). FITC fluorescence was collected through a 488-nm blocking filter, a 550-nm long-pass dichroic plus a 525-nm band pass. Measurements were amplified logarithmically.

The expression of surface P-selectin (FITC fluorescence) was analyzed in 5,000 platelets identified and gated by their specific features of size (FS) and granularity (SS) in the flow cytometer (5). Experiments were carried out in duplicated samples from four different rat donors.

Statistical analysis. All data are expressed as means ± SE. The data within groups were compared using a paired Student's t-test. An unpaired Student's t-test was used to compare between groups. Statistical significance was set at P < 0.05.

Materials. ET-1 was from American Peptide. The antibodies RMP-1 and RP-2 were made as previously described (35). Ruthenium red, CMP 48/80, mouse IgG1, and goat anti-mouse IgG1 FITC (Fc specific) mAbs were purchased from Sigma Chemical, St. Louis, MO.

RESULTS

Table 1 summarizes the results of systemic blood pressure responses before and 10 and 30 min after ET-1 administration in untreated animals and animals that were pretreated with either the P-selectin blocking antibody (RMP-1) or the binding, nonblocking control antibody (RP-2). Superfusion of the rat mesentery with ET-1 had no significant effect on systemic blood pressure at either 10 or 30 min. Moreover, administration of RMP-1 or RP-2 caused no systemic effects on blood pressure. Clearly, the hemodynamic and leukocytic effects observed in this study were localized to the mesenteric microvasculature.

Preliminary experiments revealed that both 1 and 10 nM ET-1 caused profound increases in leukocyte rolling and adhesion; however, the higher concentration induced a very large (~70%) decrease in V mean (1.4 ± 0.2 vs. 0.4 ± 0.1 mm/s) and shear rates (367 ± 58 vs. 112 ± 23 s −1) at 15 min and these values never returned to control. On the other hand, the lower concentration of ET-1 reduced shear rates by only 30% and this response was very transient, returning to control values within 10 min (Fig. 1). For this reason, the remainder of the experiments was performed at 1 nM. Although the concentration in the superfusate is 1,500-fold higher than the normal plasma levels of ET-1, on the basis of the minor hemodynamic effects, it suggests that much less reaches the vasculature.

Table 1. Systemic blood pressure measurements after 10- and 30-min superfusion with ET-1

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<th></th>
<th>Basal</th>
<th>10 Min</th>
<th>30 Min</th>
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<tr>
<td>ET-1</td>
<td>95.8 ± 5.1</td>
<td>100.8 ± 6.4*</td>
<td>101.3 ± 5.5*</td>
</tr>
<tr>
<td>RP-2 + ET-1</td>
<td>93.7 ± 5.7</td>
<td>89.3 ± 7.1*</td>
<td>91.2 ± 7.7*</td>
</tr>
<tr>
<td>RMP-1 + ET-1</td>
<td>98.5 ± 2.1</td>
<td>98.5 ± 1.7*</td>
<td>99.4 ± 2.0*</td>
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Values are means ± SE of mean arterial blood pressure in mmHg. Animals were either untreated or pretreated with nonblocking (RP-2) and blocking anti-P-selectin (RMP-1) monoclonal antibodies at 2.5 mg/kg iv. ET-1, endothelin-1 (1 nM). *No significant differences between groups.

Fig. 1. Effect of endothelin-1 (ET-1) superfusion on hemodynamic parameters. Shear rate (top), arteriolar diameter (middle), and venular diameter (bottom) were determined at 0, 10, and 30 min after ET-1 superfusion (1 nM) in animals untreated or pretreated with nonblocking (RP-2) and blocking (RMP-1) anti-P-selectin monoclonal antibodies (mAbs) (2.5 mg/kg iv, 5 min before ET-1 superfusion). Results represent means ± SE for n = 4–5 animals. *P < 0.05 relative to own control value (time 0).

less reaches the vasculature. Figure 1 also demonstrates that 1 nM ET-1 caused a significant but transient decrease in arteriolar diameter and had little effect on venular diameters.

The leukocyte rolling responses to ET-1 are illustrated in Fig. 2. Baseline leukocyte rolling responses
were 20 cells/min and the flux increased to more than 75 cells/min after a brief 2-min ET-1 superfusion and then returned to near control values by 30 min. Addition of the nonblocking control antibody had absolutely no effect on the ET-1 response at either 10 or 30 min. Addition of the anti-P-selectin antibody (RMP-1) had no effect on baseline leukocyte rolling flux values, consistent with previous observations in the rat mesentery with this and other anti-P-selectin antibodies (16, 20). However, the induction of leukocyte rolling with ET-1 was entirely inhibited by RMP-1. The bottom panel in Fig. 2 illustrates that despite profound increases in the number of rolling leukocytes associated with ET-1 leukocyte rolling velocity was not altered. Moreover, RP-2 had no effect on the rolling velocity under baseline conditions or after ET-1 superfusion. RMP-1 had no effect on the baseline velocity of rolling cells, and although the value after ET-1 + RMP-1 administration was high (126.1 ± 66.5 µm/s) relative to ET-1 alone (33.9 ± 10.2 µm/s), the variability (range: 29–322 µm/s) prevented this value from reaching significance.

Figure 3 summarizes the adhesion data. Similar to the rolling flux pattern leukocyte adhesion increased rapidly after ET-1 superfusion. By 30 min, however, the adhesion had dissipated suggesting a reversible effect of ET-1 on the firm adhesion of leukocytes to endothelium. RMP-1 but not its nonblocking counterpart, RP-2, completely prevented the firm adhesion associated with ET-1 superfusion consistent with the view that increased leukocyte rolling is required for subsequent adhesion. Interestingly, ET-1 superfusion could not elicit leukocyte emigration in postcapillary venules (Fig. 3, bottom) suggesting that ET-1 was strictly a proadhesive but not a chemotactic mediator.

ET-1, at the two different doses assayed, caused no platelet-endothelial cell interactions during the development of the different in vivo protocols. Furthermore, flow cytometry analysis on rat platelets revealed that whereas ADP causes a clear P-selectin expression (Fig. 4, top), ET-1 at 1 or 100 nM was unable to elicit any
P-selectin expression on rat platelets (Fig. 4, middle and bottom).

ET-1 consistently caused a reduction in venular blood flow and shear rates for approximately 5–10 min at which point blood flow began to return to control values. To ensure that the 10-min reduction in blood flow was not sufficient to induce the rolling and adhesion responses, blood flow was mechanically reduced (cuff rather than ET-1) for an equivalent period of time. Figure 5 demonstrates that ET-1 had an order of magnitude greater effect on leukocyte rolling than a mechanical reduction in blood flow per se (57 ± 20 vs. 6 ± 5 cells/min above baseline). Similarly, adhesion was also much greater with ET-1 than with mechanical reduction in blood flow, suggesting that the reduced hemodynamic parameters were unable to explain the leukocyte responses to ET-1.

Previous work from our laboratory has demonstrated that mast cells closely apposed to the mesenteric microvasculature can evoke very profound increases in leukocyte recruitment. To ensure that ET-1 was not activating mesenteric mast cells, ruthenium red was coadministered with ET-1 in some preparations. Mast cell activation is associated with opening of pores and increased uptake of ruthenium red as previously described by us and others (9, 26). Table 2 summarizes that ET-1 at 1 nM had no effect on mast cell reactivity, but addition of the mast cell degranulating agent CMP 48/80 at the end of each experiment was able to dramatically induce ruthenium red uptake in all mast cells.

![Fig. 4. Expression of P-selectin in platelets from rat whole peripheral blood. Panels show overlaid fluorescence (P-selectin) histograms of nonstimulated and agonist-treated platelets, as follows: (top) 80 µM ADP; (middle) 1 nM ET-1; (bottom) 100 nM ET-1. Mean fluorescence intensities are given in brackets. Histograms are representative of 4 separate experiments.](image)

![Fig. 5. Effect of 10 min ET-1 (1 nM) superfusion or 10 min ischemia on changes in leukocyte rolling flux (top) and adhesion (bottom). Results are expressed as changes in both parameters and calculated after subtraction of individual basal levels from the absolute numbers obtained. Results represent means ± SE for n = 4 animals. *P < 0.05 compared with responses between different groups.](image)

Table 2. On-line measurements of mast cell activation in ET-1-treated and CMP 48/80-treated mesenteric preparation

<table>
<thead>
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<th>% Maximum Intensity</th>
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<tr>
<td>ET-1 (1 nM)</td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>2.8 ± 1.1</td>
</tr>
<tr>
<td>10 min</td>
<td>3.8 ± 1.2</td>
</tr>
<tr>
<td>30 min</td>
<td>3.8 ± 1.2</td>
</tr>
<tr>
<td>CMP 48/80, µg/ml</td>
<td>40.4 ± 3.8*</td>
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Values are means ± SE % maximum intensity. All color images were converted to digitized gray scales and phase inverted. As mast cells became activated, they took up more stain and the intensity of stain increased. Computer program discerned 255° of gray (from absolute black to absolute white). CMP 48/80, compound 48/80. *P < 0.05, relative to own control value (time 0).
cells. These data suggest that ET-1 functions via a mast cell-independent mechanism of leukocyte recruitment. To add further credence to this contention, mast cells were stabilized as previously reported and then ET-1 was applied to the mesentery. An identical response to ET-1 was observed in the presence and absence of mast cell stabilizer, the only difference being a higher baseline rolling in the unstabilized preparations.

**DISCUSSION**

In the present study we have demonstrated that 1 nM ET-1 within 10 min causes a significant increase in leukocyte rolling and adhesion returning to basal levels 30 min later (Figs. 2 and 3). The actions of ET-1 may be mediated via a direct action on vascular endothelium as the rapid upregulation of leukocyte rolling is consistent with increased endothelial P-selectin expression and the P-selectin antibody entirely abrogated this response. Although ET-1 could conceivably have a direct effect on leukocytes, this seems unrealistic for rolling because rolling molecules (L-selectin, PSGL-1) are constitutively expressed on leukocytes, and it is the endothelial ligands that are subject to upregulation. In contrast, the firm adhesion effect of ET-1, noted in our study, may be a direct action of leukocytes. Espinosa et al. (7) have shown an upregulation of CD18 integrins on rabbit neutrophils on ET-1 stimulation. However, even this observation has been challenged because it has recently been demonstrated by Boros et al. (3) that neutrophils from animals pretreated with an iv dose of ET-1 that was proadhesive had no significant increase in the expression of CD11b/c integrins. Our data show for the first time that, despite the proadhesive effects in our system with ET-1, the peptide lacked chemotactic activity because no emigration was noted. To date mediators such as platelet-activating factor, leukotriene B4 (LTB4), N-formyl-methionyl-leucyl-phenylalanine, and C5a known to increase CD18 expression and avidity also have been shown to induce rapid leukocyte emigration in our system. ET-1 clearly functions differently than other known proadhesive molecules.

The effects of ET-1 on endothelial P-selectin expression have, to our knowledge, not previously been reported. In this context, it has been found that ET-1 causes expression of von Willebrand factor (vWF) by endothelial cells (12). P-selectin is colocalized with vWF in Weibel-Palade bodies of endothelial cells (2), and so increased vWF release is consistent with increased P-selectin expression. However, one might anticipate that increased vWF release might result in increased platelet aggregation and adhesion. In our study this was not the case because no increase in platelet-platelet or platelet-endothelium interactions could be seen in vessels treated with ET-1. Furthermore, flow cytometry analysis shows that 1 nM or 100 nM ET-1 did not induce P-selectin upregulation in rat whole blood platelets. These data are entirely consistent with our previous work with LTC4, H2O2, or even desmopressin. We documented that all of these mediators mobilize P-selectin yet in each case only leukocytes but not platelets were seen to interact with endothelium (18, 20, 21). Because intravital microscopy has been extremely effective at detecting platelet-platelet, platelet-endothelium, and platelet-leukocyte interactions, it is our view that ET-1 over a 100-fold concentration (10–0.1 nM) does not affect platelets in vivo in the rat.

However, in direct contrast, previous studies have provided evidence that ET-1 can enhance platelet aggregation evoked by ADP (23) or induce ex vivo platelet activation through elevation of intracellular calcium concentrations and expression of P-selectin (11). In vivo, ET-1-induced thrombus formation has been described in mesenteric microvessels (13). Additionally, trapping of platelets within the pulmonary circulation was detected after intravenous infusion of ET-1 (14). In our study, however, ET-1 at the different doses assayed had no effect on platelet aggregation during the course of the experiments performed within the rat mesenteric microvessels. One possible explanation for the lack of effect of ET-1 on platelets in this in vivo system is the potential release of antiaggregatory mediators such as NO or PGI2 from endothelial cells, which would prevent this effect. Indeed, ET-1 exerts its actions through its interaction with two specific receptor subtypes namely ETa and ETb receptors. ETa receptor is primarily located in the vascular smooth muscle and its stimulation results in vasoconstriction. ETb receptor can be found in both endothelial cells and in the vascular smooth muscle. Whereas interaction of ET-1 with ETb receptor present on vascular smooth muscle results in vasoconstriction, its interaction with endothelial ETb causes vasodilatation because of the release of NO and prostacyclin (8). Because both NO and prostacyclin are antiaggregatory for platelets, it is possible that this masks the proaggregatory effects of endothelin. In support of this idea, McMurdo et al. (27) found that ET-1-induced vasopressor response in the rabbit is primarily mediated via its interaction with ETA receptor, whereas the depressor and antiaggregatory actions observed are associated with ETB receptor stimulation. Different concentrations of ET-1 could therefore produce proaggregatory or antiaggregatory results and explain discrepancies among the studies. Indeed, Halim et al. (11) found that ET-1 induced platelet activation at 1 μM or higher doses, 1,000-fold higher than those used in our study, whereas McMurdo et al. (27) found antiadhesive effects at lower concentrations.

The most intriguing observation to us was the ability of ET-1 to induce leukocyte rolling in mesenteric preparations where mast cells were not stabilized before surgery. This is in stark contrast to histamine, LTC4, and H2O2, which all had no effect on P-selectin expression if the mast cells were not stabilized before exteriorization of the mesentery. This was thought to be caused by a tachyphylactic response because exteriorization of the mesentery could activate mast cells to release mediators that induced P-selectin expression, and a second P-selectin inducer would then have a minimal effect. Indeed in mast cell-stabilized preparations the first dose of histamine induced a large increase in P-selectin-dependent rolling, but the response to a
second dose was dramatically blunted. ET-1 evoked effects regardless of whether the mast cells were stabilized or not, suggesting that mast cell degranulation was not able to produce tachyphylaxis in response to ET-1. These data suggest that the mechanism by which ET-1 stimulates P-selectin is very different from that of either histamine or LTC4.

An alternative mechanism of action of ET-1, unlike histamine and LTC4, may be direct activation of mast cells to release mediators. However, we saw no activation of mast cells during ET-1 superfusion by means of ruthenium red uptake. To further confirm this assessment, a group of animals were pretreated with cromolyn, and an identical leukocyte response for ET-1 was obtained in the animals not receiving cromolyn. Our data clearly suggest no role for mast cells in ET-1-induced leukocyte rolling and adhesion. Although there is evidence that ET-1 can induce mast cell activation in the mouse (Sa, 33, 34), in studies carried out in rat peritoneal mast cells ET-1 failed to induce histamine release (4), raising the possibility of species- and/or receptor subtype differences.

At the doses of ET-1 used in this study via local application, no systemic blood pressure changes were noted; however, arteriolar diameter, blood flow, and shear rate were significantly reduced within the mesentery. Therefore, to address the possibility that ET-1-induced leukocyte-endothelial cell interactions were caused by decreased blood flow and shear rate, a transient reduction of both parameters was performed in another group of animals using an occluder. As shown in Fig. 5, whereas ET-1 superfusion resulted in a significant increase in leukocyte rolling flux and adhesion, none of these parameters was increased after 10 min of ischemia, making low flow an implausible explanation for the ET-1-observed responses. Indeed, it is well documented that at least 20 min of ischemia are necessary to observe elevated leukocyte adhesion during the reperfusion period (25).

Interestingly, it has recently been reported that ET-1-induced leukocyte rolling and adhesion in rat intestinal microcirculation is mainly mediated by its interaction with the ET_A receptors present on vascular smooth muscle because antagonists directed against this receptor subtype were able to abrogate these events, whereas an antagonist against ET_B receptor had only a minimal effect (3). In agreement with the above-mentioned report, it was previously demonstrated by Miura et al. (28) that lipopolysaccharide-induced leukocyte rolling and adhesion could be attenuated by an antagonist directed against ET_A receptor subtype. Therefore it can be postulated that ET-1, unlike histamine, LTC4, and H2O2, elicits P-selectin expression through interaction with its ET_A receptor on smooth muscle cells. This would require communication between smooth muscle cells and endothelium to induce an increase in leukocyte rolling and adhesion. This suggests for the first time that smooth muscle cells may be a critical aspect of the ET-1-induced rolling. Indeed, in a series of rolling studies carried out in vitro in a flow chamber system with cultured human umbilical vein endothelial cells stimulated with ET-1 no rolling was detected (data not shown). Static assays have also revealed no proadhesive effect of ET-1 directly on endothelium (22, 32).

In conclusion, we have provided direct evidence for the first time that ET-1 is a potent inducer of leukocyte rolling within the rat mesenteric microcirculation via rapid P-selectin expression on the vascular endothelial cell surface. The increased rolling transiently translated into increased adhesion, but ET-1 on its own was not able to induce leukocytes to emigrate. The enhanced leukocyte-endothelium interactions were not caused by their vasoconstrictor activity because an identical duration of mechanically induced ischemia did not account for the adhesive interactions elicited by ET-1. Finally, the responses observed were not induced by mediators released from activated mast cells. These data raise the possibility that the elevated levels of ET-1 noted in different cardiovascular diseases, in addition to causing hemodynamic disturbances, may also impact on enhanced leukocyte-endothelium interactions potentially enhancing inappropriate inflammatory responses in the vasculature.

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