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Characterization of Thrombin-Induced Leukocyte Rolling and Adherence: A Potential Proinflammatory Role for Proteinase-Activated Receptor-4

Nathalie Vergnolle, Claudia K. Derian, Michael R. D’Andrea, Martin Steinhoff, and Patricia Andrade-Gordon

It is commonly accepted that thrombin exerts its proinflammatory properties through the activation of proteinase-activated receptor (PAR)-1, although two other thrombin receptors have been discovered: PAR-3 and PAR-4. In this study, we have investigated the mechanisms and the receptors involved in thrombin-induced leukocyte/endothelial cell interactions by using selective agonists and antagonists of thrombin receptors in an in vivo intravital microscopy system. Topical addition of selective PAR-1 agonists to rat mesenteric venules failed to reproduce the increased leukocyte rolling and adherence observed after thrombin topical addition. When added together with the selective PAR-1 antagonist RWJ-56110, thrombin was still able to provoke increased leukocyte rolling and adherence. The thrombin-induced leukocyte rolling and adherence was not affected by pretreatment of rats with an anti-platelet serum. Selective PAR-4-activating peptide was able to reproduce the effects of thrombin on leukocyte rolling and adherence. Intrapерitoneal injection of PAR-4-activating peptide also caused a significant increase in leukocyte migration into the peritoneal cavity. In rat tissues, PAR-4 expression was detected both on endothelium and isolated leukocytes. Taken together, these results showed that in rat mesenteric venules, thrombin exerts proinflammatory properties inducing leukocyte rolling and adherence, by a mechanism independent of PAR-1 activation or platelet activation. However, PAR-4 activation either on endothelial cells or on leukocytes might be responsible for the thrombin-induced effects. These findings suggest that PAR-4 activation could contribute to several early events in the inflammatory reaction, including leukocyte rolling, adherence and recruitment, and that in addition to PAR-1, PAR-4 could be involved in proinflammatory properties of thrombin.


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The mechanism of action of thrombin has been shown to be mediated in part, via the proteolytic activation of cell-surface G-protein-coupled receptors (1–4). Thrombin acts as a proteinase, cleaving and unmasking the N-terminal amino acid sequence of the G-protein-coupled receptors and this new N-terminal domain acts as a tethered self-activating ligand. Three members of the proteinase-activated receptor (PAR) family have been described as receptors activated by thrombin: PAR-1, PAR-3, and PAR-4. These thrombin receptors have distinct tethered ligand sequences (SFLLRNPN... for human PAR-1, TFRGAPPN... for human PAR-3, and GYPGKF-NH2... for human PAR-4) (1, 3–5). A remarkable property for PAR-1 and PAR-4 (but not PAR-3) is that synthetic peptides based on the proteolytically revealed sequence (e.g., SFLLR-NH2 for PAR-1 and GYPGQV-NH2 for PAR-4) are able to activate the receptor, mimicking the actions of thrombin (3, 6). Nonetheless, it has been shown that the PAR-1-activating peptide (AP) SFLLR-NH2 (SF-NH2) is not selective for PAR-1 but is also able to activate PAR-2, a PAR triggered by trypsin or tryptase, but not thrombin (3, 5–8). Because the synthetic peptide corresponding to the tethered ligand of PAR-1 (SF-NH2) is not a selective agonist for PAR-1 but also activates PAR-2, selective PAR-1-APs have been developed: TFLLR-NH2 (TF-NH2) (3, 6, 8) and aparáfluoroFRCyclohexylACitY-NH2 (Cit-NH2) (3, 9), which are both highly selective for PAR-1. These latter two peptides can be used to reproduce the PAR-1-mediated effects of thrombin in vivo, without concurrently activating PAR-2.

The discovery of PAR-1, PAR-3, and PAR-4 resulted from a successful search for the receptors responsible for the cellular actions of thrombin on platelet aggregation (2–5). PAR-1 and PAR-4 were identified as the receptors responsible for thrombin-induced platelet activation in humans (5), while in rodents PAR-3 and PAR-4 exert the same role (5). Therefore, it is widely accepted that PAR-1, PAR-3, and PAR-4 play a key physiological role in hemostasis. Although other physiological roles have been suggested for PAR-1, especially in the setting of inflammation, no potential functions other than platelet aggregation have been identified for PAR-3 and PAR-4. However, PAR-3 appears to act only as a cofactor in the activation of PAR-4 by thrombin (11). The tethered ligand for PAR-4, GYPGKF-NH2, selectively activates PAR-4, but its lack of potency renders it of limited utility (12). A structure analysis of PAR-4 peptides has highlighted the peptide AYPGKF-NH2 as a potent and selective PAR-4 agonist, identifying this peptide as a useful tool for probing PAR-4 functions (12).
Although well recognized for its role in the coagulation cascade, thrombin also exhibits numerous proinflammatory properties (increased vascular permeability, mast cell degranulation, neutrophil chemotaxis, cytokine release, etc.). One of the critical events during inflammatory reactions is the recruitment of inflammatory cells to the site of inflammation. The ability of leukocytes to recognize vascular endothelium, to adhere to vessel wall and to transmigrate into the site of inflammation represents one of the early steps of the inflammatory reaction. Thrombin is able to induce leukocyte rolling and adhesion to the vascular endothelium (13) supposedly by a mechanism involving PAR-1 activation. In fact, an intravital microscopy study by Zimmerman et al. (14) showed that the PAR-1-AP SF-NH₂ was able to reproduce the effects of thrombin on leukocyte rolling and adhesion. Other studies have shown that PAR-1-AP can reproduce the effects of thrombin on increased adhesion molecule expression (15, 16). However, all these studies have used the nonselective PAR-1-AP SF-NH₂ to investigate the potential physiological role of PAR-1. We know now that the peptide SF-NH₂ is not selective for PAR-1 but can also activate PAR-2. We have shown that PAR-2 activation (with selective PAR-2 agonists) leads to an increase in leukocyte rolling and adhesion (17). Selective PAR-2-AP SLGRL-NH₂ also caused an increased expression of adhesion molecules (18). It is thus possible that the effect of SF-NH₂ on leukocyte rolling and adhesion observed by Zimmerman et al. (14), was due to the activation of PAR-2 instead of a selective activation of PAR-1. To understand the mechanisms by which thrombin induced leukocyte rolling and adhesion, we have used selective PAR-1-APs (TF-NH₂ and Cit-NH₂) and a PAR-1 antagonist in an intravital microscopy system. We also wanted to determine whether platelet aggregation was responsible for the thrombin-induced leukocyte rolling and adhesion. In addition, we investigated the ability of a PAR-4 selective agonist to reproduce the effects of thrombin on leukocyte rolling and adhesion. We further identified the presence of PAR-4 on leukocytes and the proinflammatory signals of PAR-4 activation.

Materials and Methods

Chemicals

All peptides were obtained from the Peptide Synthesis Facility of the University of Calgary (Calgary, Alberta, Canada; Dr. D. McMaster, Director). Thrombin was obtained from Sigma-Aldrich (St. Louis, MO). Anti-rat platelet serum was obtained from Cedarlane Laboratories (Hornby, Ontario, Canada). The PAR-1 antagonist RWJ-56110 was provided by Johnson and Johnson Pharmaceutical Research and Development (Spring House, PA), and has been fully described elsewhere (19).

Animals

Male Wistar rats (175–200 g) were obtained from Charles River Breeding Laboratories (Montreal, Quebec, Canada). Animals had free access to food and water. All experimental procedures were approved by the Animal Care Committee of the University of Calgary and were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

Leukocyte rolling and adherence in vivo

Rats (n = 6 per group) were fasted 15 h before the beginning of the experiment. They were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and a midline abdominal incision was made. As described previously, the animals were prepared for in vivo microscopic observation of the mesentery (17). Rats were placed in a supine position on an adjustable microscope stage and the mesentery was exposed over an optically clear viewing pedestal that allows for transillumination of a 2-mm² segment of mesenteric tissue. The exposed mesentery was constantly superfused with a warm bicarbonate-buffered saline, pH 7.4. The mesenteric microcirculation was observed using a microscope (Nikon Optiphot-2, Melville, NY) with a x25 objective lens (Leitz Wetzlar L25.0.35; Berlin, Germany). Single unbranched mesenteric venules (20–40 μm in diameter) were selected for the study. Images of the selected venule were recorded for 5 min, after a 15-min equilibration period, and the end of this 5-min interval was considered as time 0 (17). For the remainder of the experiment, the drugs were added to the superfusion buffer: thrombin 0.5 U/ml, control peptide inactive on PAR-1 (FS-NH₂), selective PAR-1-APs (TF-NH₂, Cit-NH₂), PAR-1/PAR-2-AP (SF-NH₂), PAR-2-AP (AYPKGF-NH₂), and the control peptide inactive on PAR-4 (FKGPYA-NH₂) 50 μM each. The PAR-1 selective antagonist RWJ-56110 was added to the superfusion buffer (10 and 30 μM) at a dose known to fully inhibit thrombin-induced PAR-1 activation (19) 10 min before starting thrombin superfusion. Complementary experiments showed that addition of RWJ-56110 to the superfusion buffer did not change the number of rolling and adherent leukocytes to the vessel wall in all experimental conditions. The images were recorded for 5-min intervals beginning at 15, 30, 45, and 60 min after the beginning of the superfusion with the drugs. Venular diameter was measured online using a video caliper (model 908; IPM, San Diego, CA). Leukocyte adherence was determined upon video playback, on 100-μm vessel length, a leukocyte being considered adherent to the endothelium if it remained stationary for 30 s or more. Leukocyte flux was defined as the number of leukocytes per minute moving at a velocity less than that of erythrocytes, which passed a reference point in the venule. The changes in flux of rolling leukocytes were evaluated as differences between the number of rolling leukocytes at each interval and the basal number of rolling leukocytes.

To evaluate the role of platelets in thrombin-induced increase in leukocyte rolling and adherence, selected groups of rats were pretreated with an anti-platelet Ab (0.5 ml/kg, from Cedarlane Laboratories) injected i.v. 20 min before the beginning of the experiment (20).

Leukocyte extravasation

Groups of rats received a 1-ml i.p. injection of the PAR-4-AP AYPKGF-NH₂, at doses from 1 to 100 μg/rat, the control peptide FKGPYA-NH₂ (100 μg/rat), or PBS, the vehicle control for those 5 experiments. Six and 24 h after this i.p. injection, the animals were sacrificed and peritoneal cavities were opened and washed with 10 ml of PBS + EDTA (3 mM) + heparin (50 U/ml), and lavage fluids were carefully collected. The lavage fluids were centrifuged for 5 min at 1200 rpm and the pellets were resuspended in 5 ml of PBS + EDTA (3 mM). The number of extravasated leukocytes was quantified by staining the lavage fluids with Turk’s solution and counting the cells present in the lavage fluids with a Neubauer hemacytometer.

Leukocyte isolation

Polymorphonuclear leukocytes (PMNs) were isolated from rat blood collected in sodium citrate (0.38% final concentration) using the method of Boyum (21). PMNs were separated on a density gradient (lymphocyte separation medium; ICN Biomedicals, Aurora, OH) followed by 2% dextran sedimentation to remove the majority of RBCs (Amersham Pharmacia Bio-Tech, Piscataway, NJ). Contaminating RBCs were lysed in 0.15 M ammonium chloride solution. Cells were washed in HBSS and then fixed in 10% neutral-buffered formalin.

Immunohistochemistry

Rat aorta tissue was routinely obtained, fixed in 10% neutral-buffered formalin, processed for paraffin embedding, cut (5 μm) onto microscopic slides, and then deparaffinized and hydrated. Isolated rat PMNs were spun onto microscopic slides using a Cyto-Tek cytosip (Torrance, CA). Tissues and cells were processed for routine immunohistochemistry as previously described (22). Briefly, slides were microwaved in target buffer (DAKO, Carpinteria, CA), cooled, placed in PBS (pH 7.4), and treated with 3.0% H₂O₂ for 10 min. All subsequent reagent incubations and washes were performed at room temperature. Normal blocking serum (Vector Laboratories, Burlingame, CA) was placed on all slides for 10 min. After briefly rinsing in PBS, primary Abs were placed on slides for 30 min. The PAR-4 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) has been previously characterized (23). Other polyclonal Abs included anti-myeloperoxidase (DAKO) and anti-factor VIII (DAKO). The slides were washed and goat anti-rabbit biotinylated secondary Abs were placed on the tissue sections for 30 min (Vector Laboratories). After rinsing in PBS, the avidin-HRP-biotin complex reagent (Vector Laboratories) was added for 30 min. Slides were washed and treated with the chromogen 3,3′-diaminobenzidine (Bio-media, Foster City, CA) twice for 5 min each, then rinsed in dH₂O, and counterstained with hematoxylin. The negative controls included replacement of the primary Ab with preimmune serum or with the same species IgG isotype nonimmune serum.
Results
Effects of thrombin and PAR-1 agonists on leukocyte rolling and adherence

As previously described (14), superfusion of rat mesenteric venules with thrombin (0.5 U/ml) significantly increased the flux of rolling leukocytes from 30 to 60 min after the addition of thrombin (Fig. 1A), while inactivated thrombin (boiled for 10 min), had no effect on leukocyte rolling. Under basal conditions (time 0), an average of three leukocytes per 100-μm vessel length were adherent to the vessel wall of the rat mesenteric venule in all groups. From 30 to 60 min after the addition of thrombin to the superfused buffer (0.5 U/ml), the number of leukocytes adhering to the vessel wall was significantly increased. No change in leukocyte adherence was observed at all time-points after the addition of boiled thrombin to the superfusion buffer, compared with the basal period (time 0) (Fig. 1B).

At all time points after the beginning of superfusion with selective PAR-1-APs (TF-NH₂ and Cit-NH₂, 50 μM) or the control peptide FS-NH₂, no change in flux of rolling leukocytes was observed compared with basal (Fig. 2A). No effect was observed using higher doses of PAR-1-AP (100 and 200 μM, data not shown). In contrast, superfusion with the nonselective PAR-1/ PAR-2-AP SF-NH₂ caused a significant increase in the number of rolling leukocytes, from 30 to 60 min after the peptide addition (Fig. 2A). Like the control peptide FS-NH₂, the selective PAR-1 agonists TF-NH₂ and Cit-NH₂ had no effect on the number of leukocytes adhering to the vessel wall. However, SF-NH₂, which can activate both PAR-1 and PAR-2, caused a significant increase in the number of adhering leukocytes from 30 to 60 min after the peptide addition to the superfusion buffer (Fig. 2B).

Role of PAR-1 in thrombin-induced leukocyte rolling and adherence

Although selective PAR-1 agonist had no effect on leukocyte rolling and adherence (Fig. 2), we wanted to further investigate whether the effects of thrombin are mediated by the activation of PAR-1. The addition of the selective PAR-1 antagonist RWJ-56110 (10 μM) to the superfusion buffer at a concentration known to block PAR-1 activation by thrombin (19) did not reduce the effects of thrombin on flux of rolling leukocytes or on adherent leukocytes (Fig. 3, A and B). A higher concentration of the PAR-1 antagonist RWJ-56110 (30 μM) had no effect on the thrombin-induced leukocyte rolling and adhesion (data not shown).

Role of platelet activation in thrombin-induced leukocyte rolling and adherence

To investigate the potential role of platelets in thrombin-induced increase in leukocyte rolling and adherence, we pretreated a group of...
of rats with an anti-platelet antiserum (a dose known to deplete rats of their platelets). The thrombin-induced increase in leukocyte rolling and adherence was not reduced by the anti-platelet treatment, while in rats treated with the anti-platelet serum vehicle, thrombin still caused a significant increase in leukocyte rolling and adherence from 30 to 60 min after its addition to the superfusion buffer (Fig. 4, A and B).

**Effects of PAR-4-APs on leukocyte rolling, adherence, and recruitment**

Superfusion of rat mesenteric venules with the selective PAR-4-AP AYPGKF-NH₂ (50 μM) significantly increased the number of rolling leukocytes from 15 to 60 min after the peptide addition, but the control peptide FKGPYA-NH₂, inactive on PAR-4, had no effects (Fig. 5A). AYPGKF-NH₂, but not FKGPYA-NH₂, also caused a significant increase in the number of leukocytes adherent to the vessel wall, from 15 to 60 min after its addition to the superfusion buffer (Fig. 5B). The peptide corresponding to the tethered ligand receptor sequence of PAR-4 (GYPGKF-NH₂, 200 μM) caused similar effects as AYPGKF-NH₂, increasing the number of rolling and adherent leukocytes, when added to the superfusion (data not shown). By injecting the selective PAR-4-AP AYPGKF-NH₂ i.p., we investigated the effects of PAR-4 activation on PMN recruitment. Six and 24 h after the i.p. injection of AYPGKF-NH₂, a significant increase in the number of PMN extravasated into the peritoneal cavity was observed, compared with the effects of the control peptide FKGPYA-NH₂ (Fig. 6A). The effects of PAR-4-AP on leukocyte extravasation into the peritoneal cavity were dose-dependent (Fig. 6B). At the two observed time points (6 and 24 h after the i.p. injection of PAR-4-AP), >95% of the extravasated cells were PMNs, only a few mononuclear cells were observed.

**Immunolocalization of PAR-4 on endothelium and PMNs**

We have investigated the presence of PAR-4 on the two cellular actors of the rolling and adherence events: the endothelium and leukocytes. Normal rat PMNs and aorta were processed for immunohistochemistry. We observed positive PAR-4 immunolabeling (arrowheads) in the PMNs (Fig. 7A) as well as in the normal endothelium (arrowheads) and in the vascular smooth muscle cells (arrows). We did not observe any immunolabeling in the negative controls (Fig. 7, B and E). Positive myeloperoxidase immunolabeling (arrowheads) confirmed the presence of PMNs (Fig. 7C), and factor VIII immunolabeling (arrowheads) confirmed the presence of endothelial cells (Fig. 7F).

**Discussion**

Consistent with previous reports (14), we observed that thrombin superfusion of mesenteric venules in vivo caused a significant increase in leukocyte rolling and adherence and that superfusion
with the nonselective PAR-1/PAR-2 peptide SF-NH₂ was able to reproduce the same effects induced by thrombin. Nonetheless, selective PAR-1-APs (TF-NH₂ and Cit-NH₂) failed to reproduce the increased leukocyte adherence and rolling observed after superfusion with thrombin, suggesting that these effects of thrombin are not mediated by the activation of PAR-1. The ability of those selective PAR-1-APs to reach the receptor and activate it can be questioned in that system, but thrombin, a full-length protein of 37,000 kDa reaches its target to induce leukocyte rolling and adherence, thus it is unlikely that small peptides cannot find their way to their receptor to induce the same effects. It is also possible that such small peptides are degraded before they can reach the receptor, but the SF-NH₂ peptide that differs from TF-NH₂ only by the first amino acid (a serine replacing a threonine residue) is able to induce leukocyte rolling and adherence. The use of high concentrations of peptides (100 and 200 μM) should allow us to see an effect of those peptides, even if they are partially degraded. However, the selective PAR-1-APs (TF-NH₂ and Cit-NH₂) had no effect on the number of rolling and adherent leukocytes, even when high peptide concentrations were used. These data strongly suggest that the activation of PAR-1 in vivo, is not responsible for the thrombin-induced leukocyte rolling and adherence, but to completely rule out a possible involvement of PAR-1 in the thrombin-induced effect on endothelial cells/leukocyte interactions, we used a selective antagonist for PAR-1. The effects of thrombin on leukocyte rolling and adherence were unchanged in the presence of the PAR-1 antagonist RWJ-56110, used at a dose known to inhibit PAR-1-induced platelet aggregation (19). Taken together, our data indicate that PAR-1 activation is not responsible for the effects of thrombin on leukocyte rolling and adhesion to the endothelial wall. Selective activation of PAR-2 by the peptide corresponding to the tethered ligand of rat PAR-2 (SLIGRL-NH₂) has been shown to cause a significant increase in rolling and adhesion of leukocytes to the vessel wall in the same system as the one used in the present study (17). Thus, the effects of the nonselective PAR-1/PAR-2-AP SF-NH₂ can be attributed entirely to PAR-2 activation.

A role for platelets in the pathogenesis of leukocyte recruitment (rolling and adherence) to the vessel wall has recently been described (20). These findings by Salter et al. (20) suggest a major role for activated platelets in ischemia/reperfusion-induced P-selectin expression and the resultant leukocyte recruitment. Thrombin’s effects on platelet activation are well-established and the presence of PAR-3 and PAR-4, the two other “thrombin receptors”, has been described on platelets. In rodents, thrombin signals to platelets through the activation of these two receptors, but not through the activation of PAR-1 (5). As we have established that thrombin provoked leukocyte rolling and adherence to the vessel wall through a mechanism independent of PAR-1 activation, it can be hypothesized that thrombin effects on leukocyte recruitment are mediated by the activation of PAR-3 and/or PAR-4 on platelets, which in turn provoke the leukocyte/endothelial cell interaction signal. To address this issue, we monitored thrombin-induced leukocyte rolling and adhesion in mesenteric venules of untreated rats and in rats depleted of platelets by pretreatment with an anti-rat platelet Ab. The results revealed that the anti-platelet intervention did not affect the thrombin-induced increased leukocyte rolling and adherence (Fig. 4), thus showing that platelet activation was not

FIGURE 5. Effects of PAR-4 selective agonist on leukocyte rolling and adherence. Time-dependent changes in flux of rolling leukocytes (A) and adherent leukocytes (B) after the addition of the selective PAR-4-activating peptide AYPGKF-NH₂ (50 μM) or the control peptide FKGPYA-NH₂ (50 μM) to the superfusion buffer. Values are mean ± SEM of n = 6 per group. * Significantly different from control peptide, p < 0.05.

FIGURE 6. PMN recruitment in response to selective PAR-4 agonist. Effects of i.p. injection of the selective PAR-4 agonist AYPGKF-NH₂ or the control peptide FKGPYA-NH₂ on leukocyte recruitment into the peritoneal cavity, 6 and 24 h after the i.p. injection of 100 μg/mouse of each peptide (A), and 6 h after the i.p. injection of 1–100 μg/mouse of AYPGKF-NH₂ or 100 μg/mouse of FKGPYA-NH₂ (B). Values are mean ± SEM of n = 6 per group. * Significantly different from control peptide, p < 0.05.
required in the mechanism of thrombin-induced leukocyte/endothelial cell interactions. This result suggests a limited role for platelets in the proinflammatory effect of thrombin related to leukocyte/endothelial cell interactions.

PAR-4 has first been described on platelets. Its presence has also been reported on cultured smooth muscle cells (24), on lung epithelium (25) and on endothelial cells (26). We have shown in this study, for the first time, its specific expression in isolated rat leukocytes. We also confirmed its presence on rat vascular smooth muscle tissues and endothelium (Fig. 7A). It is thus possible that PAR-4 activation either on leukocytes or endothelial cells might be responsible for the thrombin-induced increase in leukocyte rolling and adherence. In this study, we have shown that the selective PAR-4-AP AYPGKF-NH₂ reproduced the effects of thrombin, inducing a significant increase in the number of leukocytes rolling and adhering to the vessel wall, from 15 to 60 min after the beginning of its superfusion on mesenteric vessels. The effect of the PAR-4-AP was more rapid (significant 15 min after its addition) than the effect of thrombin (significant only 30 min after its addition). This could not be explained by a better affinity of the peptide for the receptor since for all PARs, proteinases are known to be more potent than the peptides to activate the receptor. The ability of a small peptide to reach the receptor in this preparation might be facilitated compared with thrombin, a full-length protein. It can be noted that although the PAR-4-AP caused a significant effect at an earlier time-point than thrombin, the amplitude of the response (flux of rolling leukocytes and number of adherent leukocytes) was the same for PAR-4-AP and thrombin. Although PAR-4 can be activated endogenously by other proteinases than thrombin (trypsin, cathepsin G), the fact that PAR-4 activation reproduced the effects of thrombin in our system points to a possible role of PAR-4 in thrombin-induced leukocyte/endothelial cell interactions. Also, this last result described for the first time proinflammatory properties for selective PAR-4 agonists. AYPGKF-NH₂ was able to induce in vivo leukocyte rolling and adhesion to the endothelium, but was also able to provoke full recruitment of leukocytes, as observed by the increased leukocyte extravasation into the peritoneal cavity, 6 and 24 h after the i.p. injection of PAR-4-AP (Fig. 6). A 10- to 30-fold increase in the amount of PMNs recovered from the peritoneal lavage was observed after PAR-4-AP injection, compared with the number of cells collected after the injection of the control peptide. These results indicate that PAR-4-AP not only acts on leukocyte rolling and adherence, the first two steps of leukocyte recruitment, but is also able to induce leukocyte extravasation, allowing leukocytes to migrate to the inflammatory site. We also observed transmigration of leukocytes through the venule wall at different time-points (30, 45, and 60 min.) of the intravital microscopy experiments, while this effect was never observed after the addition of the control peptide (data not shown). The fact that PAR-4 is present both on endothelial cells and leukocytes, the two major actors of inflammatory cell recruitment, suggests that the proinflammatory effects of the PAR-4 agonist are mediated through PAR-4 activation on endothelial cells and/or leukocytes. However, we cannot rule out a possible involvement of platelet activation in PAR-4-AP-induced leukocyte rolling and adherence.

In conclusion, this study demonstrated that thrombin-induced leukocyte rolling and adhesion to the mesenteric vessel wall was not mediated by the activation of PAR-1 and did not involve platelet activation. More importantly, PAR-4, which has been described as another thrombin receptor, is present on leukocytes, endothelial cells, and smooth muscle cells, and is able to produce the effects of
thrombin on leukocyte rolling and adherence. A selective PAR-4 agonist was also able to induce extravasation and full recruitment of leukocytes, demonstrating for the first time proinflammatory properties for PAR-4. These results further characterize the proinflammatory effects of thrombin on leukocyte/endothelial cell interactions and highlight PAR-4 as a potential active mediator in the inflammatory process.

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