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

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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 adhesion 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 adhesion 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 adhesion. Intraperitoneal 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. The Journal of Immunology, 2002, 169: 1467–1473.

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 protease, 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 GYPGQQ... 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) SFLLRN-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, 6–8). 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: TFFLR-NH₂ (TF-NH₂) (3, 6, 8) and apafuorofRcyclehxy1AciY-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 (10). 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).

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1 Abbreviations used in this paper: PAR, proteinase-activated receptor; AP, activating peptide; PMN, polymorphonuclear leukocyte; SF-NH₂, SFLLR-NH₂; TF-NH₂, TFFLR-NH₂; Cit-NH₂, apafuorofRcyclehxy1AciY-NH₂.
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 adherence. Other studies have shown that PAR-1-AP can reproduce the effects of thrombin on increased adhesion molecule expression (15, 16). However, 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 adherence (17). Selective PAR-2-AP SLIGRL-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 adherence, 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 adherence. 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 John-University of Calgary (Calgary, Alberta, Canada; Dr. D. McMaster, Director). 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 adhesion 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 AYPGKF-NH₂, at doses of 1 to 100 μg/rat, the control peptide FKGPY-NH₂ (100 μg/rat), or PBS, the vehicle control. 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 Türk’s solution and counting the cells present in the lavage fluids with a Neubauer hemocytometer.

**Leukocyte isolation**

Polymorphonuclear leukocytes (PMNs) were isolated from rat blood collected in sodium citrate (0.38% final concentration) using the method of Boehringer (14). PMNs were separated on a density gradient (lymphocyte separation medium; ICN Biomedical, Aurora, OH) followed by 2% dextran sedimentation to remove the majority of RBCs (Amersham Pharmacia Biotech, 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 using a Cyto-Tek cytospin (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). 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 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 treat-
ment, while in rats treated with the anti-platelet serum vehicle,
the 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 teth-
ered ligand receptor sequence of PAR-4 (GYPGKF-NH2, 200 μM)
cause 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 im-
munohistochemistry. We observed positive PAR-4 immunolabel-
ing (arrowheads) in the PMNs (Fig. 7A), and factor VIII immunolabel-
ing (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 in-
crease in leukocyte rolling and adherence and that superfusion

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**Figure 3.** PAR-1 antagonist effects on thrombin-induced leukocyte rolling and adherence. Time-dependent changes in flux of rolling leukocytes (A) and adherent leukocytes (B) after the addition of thrombin alone (0.5 U/ml) or thrombin in the presence of the PAR-1 antagonist RWJ-56110 (10 μM) to the superfusion buffer. Values are mean ± SEM of n = 6 per group.

**Figure 4.** Effects of platelet depletion on thrombin-induced leukocyte rolling and adherence. Time-dependent changes in flux of rolling leukocytes (A) and adherent leukocytes (B) after the addition of thrombin (0.5 U/ml) to the superfusion buffer in rats pretreated or not with an anti-rat platelet serum. Values are mean ± SEM of n = 6 per group.
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 these 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 these 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 adhesion 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
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.

FIGURE 7. PAR-4 immunolabeling in rat aorta and isolated PMNs. A, PAR-4 immunodetection in PMNs (arrowheads). B, Lack of immunolabeling in negative control. C, Presence of myeloperoxidase immunolabeling (arrowheads) confirming presence of PMNs. D, Presence of PAR-4 immunolabeling in aortic smooth muscle cells (arrows) and in the endothelial cells (arrowheads). E, Lack of immunolabeling in negative control. F, Presence of factor VIII immunolabeling (arrowheads) in aortic endothelial cells. Scale bar represents 25 μm for all figures.

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 PAR-4 agonists.
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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