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Pro- and anti-inflammatory actions of thrombin: a distinct role for proteinase-activated receptor-1 (PAR_1)

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1 Thrombin has well characterized pro-inflammatory actions that have recently been suggested to occur *via* activation of its receptor, proteinase-activated receptor-1 (PAR_1).

2 In the present study, we have compared the effects of thrombin to those of two peptides that selectively activate the PAR_1 receptor, in a rat hindpaw oedema model. We have also examined whether or not thrombin can exert anti-inflammatory activity in this model.

3 Both thrombin and the two PAR_1 activating peptides induced significant oedema in the rat hindpaw following subplantar injection.

4 The oedema induced by thrombin was abolished by pre-incubation with hirudin, and was markedly reduced in rats in which mast cells were depleted through treatment with compound 48/80 and in rats pretreated with indomethacin. In contrast, administration of the PAR_1 activating peptides produced an oedema response that was not reduced by indomethacin and was only slightly reduced in rats pretreated with compound 48/80.

5 Co-administration of thrombin together with a PAR_1 activating receptor resulted in a significantly smaller oedema response than that seen with the PAR_1 activating peptide alone. This anti-inflammatory effect of thrombin was abolished by pre-incubation with hirudin.

6 These results demonstrate that the pro-inflammatory effects of thrombin occur through a mast-cell dependent mechanism that is, at least in part, independent of activation of the PAR_1 receptor. Moreover, thrombin is able to exert anti-inflammatory effects that are also unrelated to the activation of PAR_1.

Keywords: Thrombin; protease-activated receptor; inflammation; mast cell; prostaglandin; nitric oxide

Abbreviations: Cit-NH_2, AparafluoroFRCyclohexylACitY-NH_2; FS-NH_2, FSLLRY-NH_2; L-NAME, N^{ω}-nitro-L-arginine methyl ester; PAR_1, proteinase-activated receptor-1; PAR_2, proteinase-activated receptor-2; PAR_3, proteinase-activated receptor-3; PAR_4, proteinase-activated receptor-4; PAR_1APs, proteinase-activated receptor-1-activating peptides; PARs, proteolytically-activated-receptors; TF-NH_2, TFLLR-NH_2; TRAP-14, SFLLRNPNDKYEPF

Introduction

Well recognized for its role in the coagulation cascade, thrombin is now known to affect its target tissues, in part, *via* the proteolytic activation of cell surface G-protein-coupled receptors (Rasmussen *et al*., 1991; Vu *et al*., 1991; Ishihara *et al*., 1997; Xu *et al*., 1998). The unique mechanism whereby thrombin activates its G-protein-coupled receptors involves the proteolytic unmasking of an N-terminal amino acid sequence that acts as a tethered, self-activating ligand. Three of these Proteolytically-Activated-Receptors (PARs) for thrombin have now been described (PAR_1, PAR_3 and PAR_4), with distinct tethered-ligand sequences (SFLLRNPN... for human PAR_1 and TFRGAPPN... for human PAR_3; (Vu *et al*., 1991; Ishihara *et al*., 1997); GYPGQV... for human PAR_4; (Xu *et al*., 1998; Kahn *et al*., 1998). A remarkable property for PAR_1 (but not PAR_3) is that synthetic peptides based on the proteolytically-revealed PAR_1 sequence (e.g., SFLLR-NH_2), in isolation, are able to activate the receptor, so as to mimic the actions of thrombin in platelets or other target tissues (Vu *et al*., 1991; Hollenberg *et al*., 1992). The pharmacology of PAR_4 has yet to be studied in depth, but does not appear to be triggered by PAR_1-activating peptides. PAR_3, which rather than PAR_1, represents the thrombin-activated receptor in rodent platelets, is not activated by synthetic peptides based either on its own revealed tethered ligand (TFRGAP-NH_2) or on the PAR_1-activating peptides (formerly termed thrombin receptor-activating peptides, or TRAPs, such as SFLLR-NH_2). Nonetheless, PAR_1-activating peptides, like SFLLR-NH_2 are also able to activate PAR_2, a proteinase-activated receptor triggered by trypsin but not thrombin. PAR_2, which is distinct from three thrombin receptors cloned to date (Nystedt *et al*., 1994) and has a unique tethered ligand sequence (SLIGRL...). Because of the ability of the above-mentioned PAR_1-derived peptides to activate both PAR_1 and PAR_2 (Blackhart *et al*., 1996; Hollenberg *et al*., 1997), we have sought to develop selective PAR_1 activating peptides by altering the peptide sequence derived from the tethered ligand of human PAR_1. We have found that two peptides, TFLLR-NH_2 (TF-NH_2) (Hollenberg *et al*., 1997) and AparafluoroFRCyclohexylACitY-NH_2 (Cit-NH_2) (Vergnolle *et al*., 1998) are highly selective for PAR_1, compared with PAR_2, and can therefore be used as surrogates for thrombin to activate PAR_1 *in vivo*, without concurrently activating PAR_2.

Apart from causing effects on target cells *via* the proteolytic activation of the G-protein-coupled receptors, PAR_1, PAR_3 and PAR_4, thrombin also exhibits chemotactic and mitogenic activity due to two peptide sequences that lie outside its catalytic domain (Bar-Shavit *et al*., 1983; 1984; Herbert *et al*.,

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1994; Stiernberg *et al*., 1993; Glenn *et al*., 1988). These non-catalytic actions of thrombin, in addition to its ability to catalyze the formation of fibrin, may contribute to the potential role of thrombin in the inflammatory response. Previous work has shown that thrombin can cause effects that accompany an inflammatory response, including increased vascular permeability (Malik & Fenton, 1992), degranulation of mast cells (Razin & Marx, 1984), increased endothelial adhesion of neutrophils (Toothill *et al*., 1990), chemotaxis and aggregation of neutrophils (Bizios *et al*., 1986) and stimulation of cytokine release from endothelial cells (Stankova *et al*., 1995; Ueno *et al*., 1996) and from vascular smooth muscle (Kranzhofer *et al*., 1996). The precise role of the thrombin receptors (PAR_1, PAR_3 or PAR_4) in these pro-inflammatory actions of thrombin is of considerable interest. In a recent study, Hirulog was observed to attenuate the inflammatory effect of thrombin in a carrageenan-induced rat paw oedema model (Cirino *et al*., 1996). Since Hirulog (a derivative of hirudin) can bind both to the catalytic site and the anion-binding exosite of thrombin (Maraganore *et al*., 1990; Skrzypczak-Jankun *et al*., 1991), it could block the actions of thrombin mediated both by the catalytic activation of PARs and by the non-catalytic activities of the exosite mitogenic/chemotactic peptide domains. To distinguish between these alternatives, Cirino *et al*. (1996) made use of the PAR_1-activating peptide, SFLLRNPNDKYEPF (TRAP-14), to evaluate its actions in the paw oedema model in comparison with thrombin. Because TRAP-14 mimicked the oedema response elicited by thrombin, causing mast cell degranulation and increased vascular permeability, it was concluded that the pro-inflammatory effects of thrombin were due to its activation of PAR_1. However, as pointed out above, data appearing subsequent to the study of *Cirino et al*. (1996) show that TRAP-14 can activate both PAR_1 and PAR_2 (Blackhart *et al*., 1996; Hollenberg *et al*., 1997; Kawabata *et al*.,1999), and that TRAP-14 is not able to activate PAR_3 (Ishihara *et al*., 1997). Thus, the conclusion that PAR_1 activation alone accounts for the pro-inflammatory actions of thrombin, based on the inflammatory action of TRAP-14, requires a re-evaluation.

In view of the above discussion, the work we describe in this report was done with two aims in mind. First, we wished to use our newly developed PAR_1-selective activating peptides (TF-NH_2 and Cit-NH_2) to determine if they would, like TRAP-14, mimic the actions of thrombin in a rat paw oedema model of inflammation. Second, we wished to examine in further detail, the mechanism and extended time course of the inflammatory response induced by thrombin and the selective PAR_1-activating peptides (PAR_1APs). Our data show that PAR_1 activation alone cannot account for all of the effects of thrombin in this model of inflammation. Moreover, our results reveal a novel anti-inflammatory action of thrombin that has yet to be appreciated.

Methods

Animals

Male, Wistar rats (175–200 g) were obtained from Charles River Breeding Farms (Montreal, QC, Canada). The rats had free access to food and water and were housed under constant temperature (22°C) and photoperiod (12-h light-dark cycle). 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.

Paw oedema

A basal recording of paw volume was made using a hydroplethismometer (Ugo Basile, Milan, Italy). The rats were lightly anaesthetized with Halothane (5%). A subplantar injection of one of the test substances was then made ($n=5$ per group in all experiments). The test substances were always injected as a total volume of 0.1 ml. The test substances were initially dissolved in HEPES (25 mM, pH 7.4), then diluted in sterile 0.9% saline to give the desired concentration. The test substances included thrombin (1.5 and 20 U per paw), the PAR_1APs, Cit-NH_2 and TF-NH_2 (500 μg), and the control peptide FSLLRY-NH_2 (FS-NH_2; 500 μg). Paw volume was measured every hour for 6 h after the injection.

To determine whether or not any pro-inflammatory activity of thrombin was due to its proteolytic activity, additional experiments were performed in which subplantar injections of thrombin (5 U per paw), hirudin (5 U per paw) or thrombin + hirudin (5 U of each pre-incubated together for 20 min at 37°C before the injection) were performed. Hirudin binds to thrombin, inhibiting its proteolytic activity.

For the evaluation of the effects of drugs on oedema formation, some groups of rats were pretreated with indomethacin (5 mg kg^{-1}, p.o.) or N^{ω}-nitro-L-arginine methyl ester (L-NAME; 25 mg kg^{-1}, i.p.) 1 h before the subplantar injection of thrombin or a PAR_1AP. The control groups received the vehicles for indomethacin and L-NAME (5% $NaHCO_3$ and saline, respectively). Other groups of rats were treated with compound 48/80 to deplete mast cells in the paw, as described by Di Rosa *et al*. (1971). Briefly, compound 48/80 (0.1% solution in 0.9% sterile saline) was injected intraperitoneally each morning and evening for 4 days prior to the paw oedema experiment. The doses employed were 0.6 mg kg^{-1} for the first six injections and 1.2 mg kg^{-1} for the last two injections. The test substances in the paw oedema experiments were administered 5–6 h after the final injection of compound 48/80.

Effects of thrombin on PAR_1AP-induced paw oedema

Paw oedema experiments were performed, as described above, except that each rat received two injections (50 μl each) into a hindpaw. Four groups of rats ($n=5$ in each) were studied: (i) thrombin (5 U) plus vehicle, (ii) PAR-1AP (500 μg) plus vehicle, (iii) thrombin (5 U) plus PAR_1AP (500 μg), and (iv) vehicle alone (100 μl). The PAR_1AP used in these experiments was Cit-NH_2. Additional experiments were then performed in which rats received subplantar injections of Cit-NH_2 (500 μg) plus hirudin (5 U per paw) or Cit-NH_2 (500 μg) together with thrombin + hirudin (5 U of each pre-incubated together for 20 min at 37°C before the injection).

Histology

Rats ($n=6$ per group) were given a 0.1 ml subplantar injection of either the inactive peptide FS-NH_2 (500 μg), the PAR_1APs Cit-NH_2 (500 μg) and TF-NH_2 (500 μg), or vehicle and were killed 6 h later. The injected paws were removed and fixed by immersion in formalin for 24 h before being embedded in paraffin wax. Sections (5 μm) were cut and stained with haematoxylin and eosin to reveal structural features.

Materials

All peptides, prepared by solid phase synthesis, were obtained from the peptide synthesis facility of the University of Calgary Faculty of Medicine (director, Dr D. McMaster). The

composition and the purity of all peptides were confirmed by HPLC analysis, mass spectral analysis and amino acid analysis. Stock solutions prepared in HEPES buffer, pH 7.4 (25 mM), were analysed by quantitative amino acid analysis to verify peptide concentration and purity. Thrombin (EC 3.4.21.5, 1460 NIH units mg^{-1}), hirudin, indomethacin, L-NAME and compound 48/80 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Statistical analysis

All results are reported as means ± s.e.mean. Comparisons among groups were performed using the two-sided Student's *t*-test with Bonferroni correction. With all statistical analyses, an associated probability (*P* value) of less than 5% was considered significant.

Results

Oedema induced by thrombin

Injection of thrombin into the rat hindpaw resulted in the development of oedema which persisted for up to 6 h (Figure 1). At the lowest concentration tested (1 U per paw), the magnitude of the oedema response was not significantly different from that observed following injection of vehicle. With higher concentrations of thrombin (5 and 20 U per paw), a more profound and long-lasting oedema response was observed. No difference in the magnitude of the oedema was observed between the 5 and 20 U per paw doses.

To verify that the observed oedema was produced through the action of thrombin itself, we tested the effects of thrombin (5 U per paw) that had been pre-incubated with the thrombin inhibitor, hirudin (5 U). No oedema was observed after the injection of thrombin + hirudin (Figure 1A) or the injection of hirudin alone (5 U per paw; not shown).

Inflammation induced by PAR_1APs

The two selective PAR_1APs (Cit-NH_2 and TF-NH_2) each induced a significant oedema response when injected into the rat hindpaw (Figure 1B). In each case, the oedema response was significantly greater (from the second hour through the sixth hour) than that observed when the control peptide was injected. Notwithstanding, the partial reverse-sequence peptide, FS-NH_2, which is unable to activate PAR_1, did yield an oedema response between 1 and 2 h that was greater than that seen with saline alone (Figure 1B). The oedema response to Cit-NH_2 was about 2 fold greater than that observed with thrombin.

Histologic examination of paws injected with the PAR_1-APs Cit-NH_2 and TF-NH_2, revealed a complete disruption of tissue architecture and oedema, as compared with control tissue sections of rat injected with vehicle. The tissues of rat paws injected with the control peptide FS-NH_2 exhibited some disruption of tissue architecture but less marked than that observed after PAR_1APs injection. Numerous granulocytes were evident in the paws of rats injected with the two PAR_1-APs (Cit-NH_2 and TF-NH_2), but no infiltrating cells were observed in the paws of rats injected either with the inactive peptide FS-NH_2 or with vehicle.

Role of mast cells

Prior treatment with compound 48/80 resulted in an oedema response to thrombin that was no greater that the administration of saline alone (Figures 1A and 2A). The oedema observed following injection of the control peptide (FS-NH_2) was also reduced to control levels by pretreatment of the animals with compound 48/80 (Figure 2B). In contrast, compound 48/80 only produced a small reduction (significant at the fourth and fifth hour) in the oedema response to the selective PAR_1AP, Cit-NH_2 (Figure 2B).

Role of prostaglandins

Pretreatment with indomethacin abolished the oedema induced by thrombin (Figure 3A). In contrast, indomethacin pretreatment resulted in a significant increase in the oedema induced by the PAR_1AP, Cit-NH_2, and had no effect on the oedema induced by the control peptide, FS-NH_2 (Figure 3B).

Role of nitric oxide

Pretreatment with L-NAME did not significantly affect the oedema response to thrombin (Figure 4A), nor did it affect the oedema response to the Cit-NH_2, or the control peptide, FS-NH_2 (Figure 4B).

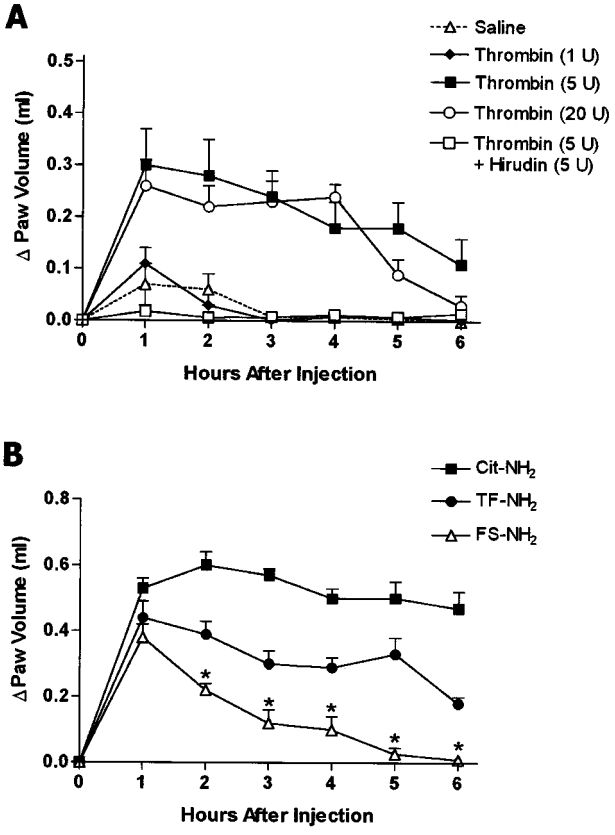

Figure 1 (A) Increase in rat hindpaw volume following injection of thrombin or thrombin that had been pre-incubated with hirudin. Thrombin caused significant oedema formation at doses of 5 and 20 U per paw. The pre-incubation of thrombin with hirudin resulted in a significant reduction in oedema at hours 1 through 5 post-injection ($P<0.05$). (B) Increase in rat hindpaw volume following injection of a PAR_1 activating peptide (Cit-NH_2 and TF-NH_2, each at 500 μg paw^{-1}) or a control peptide (FS-NH_2, 500 μg paw^{-1}). Asterisks denote significant differences ($P<0.05$) between the control peptide group and the other two groups.

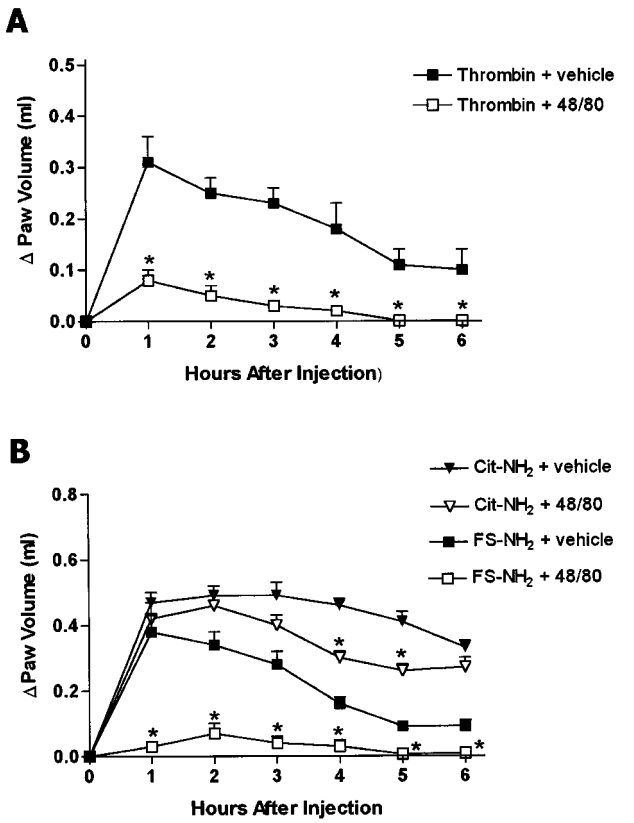

Figure 2 (A) Effects of prior depletion of mast cells (through pretreatment with compound 48/80) on the increase in hindpaw volume following injection of thrombin. The two groups differed significantly ($P<0.05$) at hours 1 through 6 post-injection. (B) Effects of prior depletion of mast cells on the increase in hindpaw volume following injection of a PAR_1 activating peptide (Cit-NH_2) or a control peptide (FS-NH_2). Asterisks denote significant differences from the corresponding group not treated with compound 48/80.

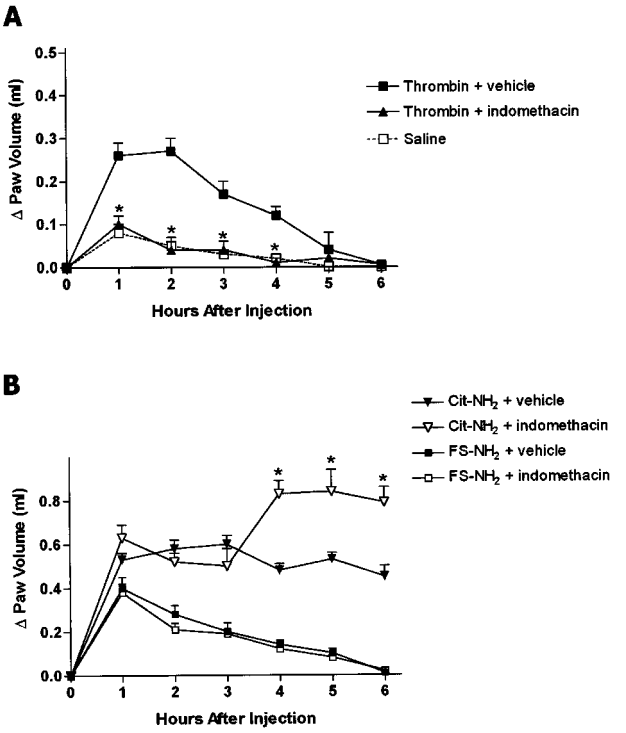

Figure 3 (A) Effects of pretreatment with the cyclo-oxygenase inhibitor, indomethacin (5 mg kg^{-1}), on the increase in hindpaw volume following injection of thrombin. Indomethacin pretreatment had no significant effect on the oedema response. (B) Effects of pretreatment with indomethacin (5 mg kg^{-1}) on the increase in hindpaw volume following injection of a PAR_1 activating peptide (Cit-NH_2) or a control peptide (FS-NH_2). Indomethacin significantly increased (*$P<0.05$) the oedema response to the PAR_1AP, while having no effect on the response to the control peptide.

Effects of thrombin + PAR_1-activating peptide

Co-administration of thrombin with the PAR_1AP, Cit-NH_2, resulted in a significant reduction (by $\sim$50%) of the oedema response compared to that produced by the PAR_1AP alone (Figure 5A). Administration of hirudin together with Cit-NH_2 had no effect on the oedema response compared to that seen with Cit-NH_2 alone. However, hirudin completely blocked the anti-inflammatory effect of thrombin (Figure 5B).

Discussion

The ability of thrombin to activate human platelets and to influence the function of numerous other cells is mediated through the proteolytic activation of a receptor that is now referred to as PAR_1 (Vu *et al*., 1991; Hollenberg, 1996). In the present study, we have demonstrated that thrombin can exert both pro- and anti-inflammatory actions, and that these actions occur, at least in part, through mechanisms distinct from the activation of PAR_1. These conclusions are based on our observation that thrombin has a different profile of activity in the paw oedema assay than two selective PAR_1APs. For example, the oedema induced by thrombin was almost completely abolished by prior depletion of mast cells (with compound 48/80) and by pretreatment with indomethacin. In contrast, the oedema induced by the PAR_1APs was slightly increased (Cit-NH_2) or not affected (TF-NH_2) by indomethacin pretreatment, and only marginally reduced in rats treated with compound 48/80. Moreover, the combined administration of thrombin and a selective PAR_1AP revealed anti-inflammatory effects of thrombin, in that the magnitude of the oedema response was significantly less ($\sim$50%) than that observed following injection of the PAR_1AP alone.

Cirino *et al*. (1996) recently reported that the pro-inflammatory effects of thrombin were mediated through activation of PAR_1. This conclusion was based on their observations that injection of TRAP-14 resulted in paw oedema similar to that seen with thrombin itself. They also demonstrated that the pro-inflammatory effects of TRAP-14 were almost completely inhibited by compound 48/80 and attenuated to some extent by indomethacin. Our observations with respect to thrombin were consistent with those of Cirino *et al*. (1996), but the observations with respect to TRAP-14 and our new PAR_1APs were divergent. These discrepancies may be explained by differences in the time frame of the two studies, and in the lack of selectivity of TRAP-14 for PAR_1 (Blackhart *et al*., 1996; Hollenberg *et al*., 1997; Kawabata *et al*., 1999). In the present study, the oedema responses were monitored for 6 h following injection of the test substances, while in the study of Cirino *et al*. (1996), the responses were monitored for only 90 min. Surprisingly, we found that a significant oedema response (compared to that observed with saline alone) could be detected during the first 1–2 h following the administration of a control peptide (FS-NH_2) that cannot activate PAR_1 (Vu *et al*., 1991; Vassallo *et al*., 1992). However, unlike after administration of PAR_1APs, no cell infiltration

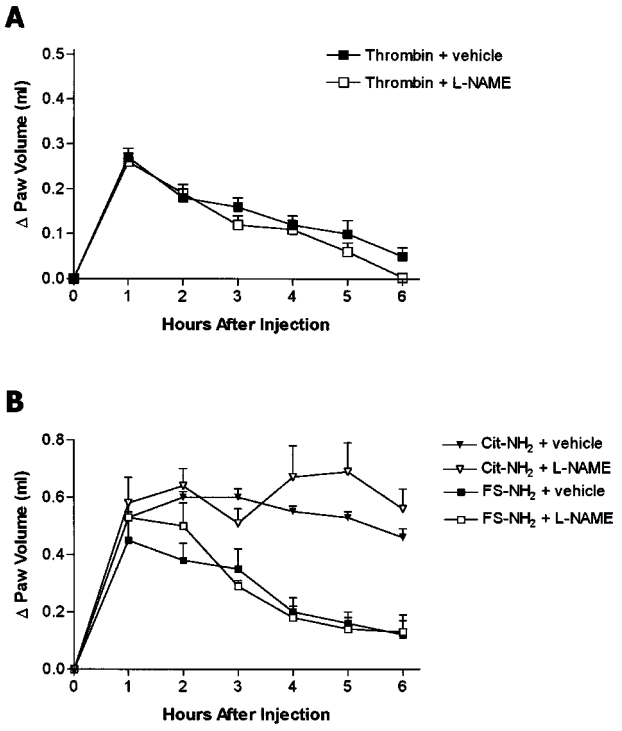

Figure 4 (A) Effects of pretreatment with the nitric oxide synthase inhibitor, L-NAME (25 mg kg^{-1}), on the increase in hindpaw volume following injection of thrombin. L-NAME pretreatment had no significant effect on the oedema response. (B) Effects of pretreatment with L-NAME (25 mg kg^{-1}) on the increase in hindpaw volume following injection of a PAR_1 activating peptide (Cit-NH_2) or a control peptide (FS-NH_2). L-NAME pretreatment had no significant effect on the oedema response.

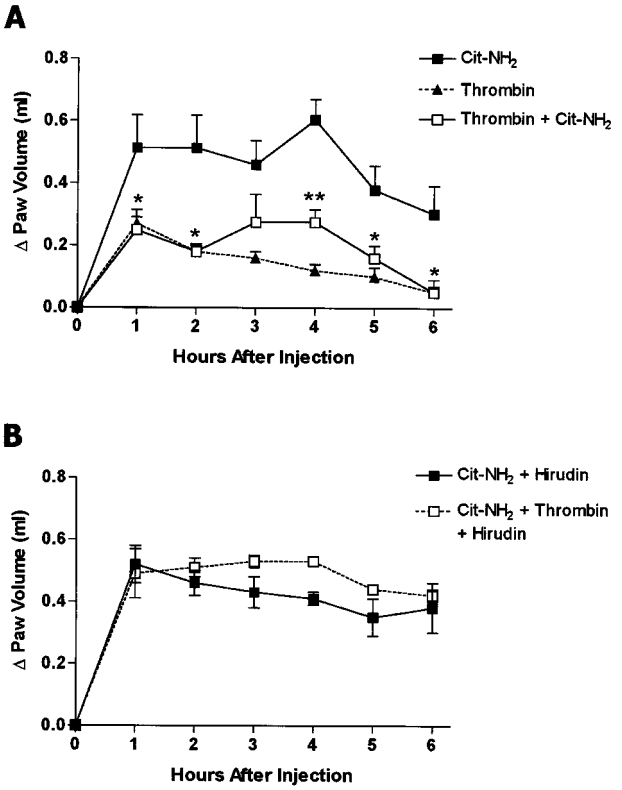

Figure 5 (A) Effects of subplantar co-administration of thrombin (5 U per paw) and the PAR-1 activating peptide (Cit-NH_2) on hindpaw volume. The oedema response following administration of thrombin and the PAR_1AP was significantly smaller than that produced by injection of the PAR_1AP alone ($^*P<0.05$ versus the group treated with PAR_1AP alone). (B) Effects of hirudin on the oedema response to the PAR_1-activating peptide, Cit-NH_2, and on the anti-inflammatory effect of thrombin observed when it was co-administered with Cit-NH_2.

was observed after the injection of the control peptide FS-NH_2. The oedema response to the partial reverse-sequence peptide was completely inhibited by compound 48/80, and was therefore likely due to activation of mast cells. It is possible that the mast cell-dependent portion of the oedema response to PAR_1APs and the reverse-sequence control peptide may have been attributable to the aromatic/basic residues in these peptides. Such residues could potentially activate mast cells in a manner similar to the activation caused by compound 48/80, a formaldehyde Schiff-base conjugate of N-methyl-O-methylphenylethylamine, that can also expose mast cells to both aromatic and basic substituents. Further, the oedema response observed in previous work employing TRAP-14 may also have been due to the concurrent activation of PAR_2. A recent study showed that PAR_2 activation by the PAR_2AP, SL-NH_2 caused an increase in vascular permeability (Kawabata *et al.* 1998). While the ability of thrombin to activate mast cells is well established (Razin & Marx, 1984), this effect may occur independent of the activation of PAR_1. It is possible that the thrombin-induced activation of mast cells may be due to the exosite peptide domains, which have previously been shown to mediate the chemotactic and mitogenic activity of thrombin (Bar-Shavit *et al.*, 1983; Glenn *et al.*, 1988).

A potential anti-inflammatory effect of thrombin was suggested by the observation that the oedema response it produced was consistently smaller in magnitude to what could be achieved with a selective PAR_1AP. As mentioned above, such an effect was confirmed by the observation that the administration of thrombin together with the PAR_1AP, Cit-NH_2, resulted in a significantly attenuated oedema response. Like the pro-inflammatory effects of thrombin, the anti-inflammatory activity was abolished by pre-incubation with hirudin. These 'anti-inflammatory' actions of thrombin may be due to its ability to activate PAR_3, PAR_4 or other PARs (Tay-Uyboco *et al.*, 1995; Ishihara *et al.*, 1997; Kahn *et al.*, 1998; Vergnolle *et al.*, 1998; Xu *et al.*, 1998) that may be present in the paw tissue. Alternatively, it is possible that the 'anti-inflammatory' effects of thrombin are due to the activity of the noncatalytic exosite peptide domains on the molecule discussed above (Bar-Shavit *et al.*, 1983; 1984; Glenn *et al.*, 1988; Herbert *et al.*, 1994; Stiernberg *et al.*, 1993, Hollenberg *et al.*, 1996).

Our results clearly show that PAR_1 activation in the rat paw displays the two main features of inflammatory process: a vascular response characterized by an oedema and granulocyte infiltration. However, the mechanism of action of the selective PAR_1APs in terms of inducing oedema formation is not clear. PAR_1 activation has been shown to result in the liberation of arachidonic acid and eicosanoids (Bills *et al.*, 1977; Weksler *et al.*, 1978; Kramer *et al.*, 1993; Bartoli *et al.*, 1994), which could contribute to the inflammatory response. However, the lack of an inhibitory effect of indomethacin on the oedema observed following PAR_1AP administration suggests that the effect was not mediated *via* a prostanoid. PAR_1 activation can also result in liberation of nitric oxide (Draijer *et al.*, 1995), but pretreatment with the nitric oxide synthase inhibitor, L-NAME, failed to modify the oedema response to the selective PAR_1APs. Since only enzymatically active thrombin has been observed to increase vascular endothelial permeability (Malik

& Fenton, 1992), and since PAR_1 receptors are present on the endothelial cell (Nelkin *et al*., 1992), it is possible that the vascular endothelium represents the target for the PAR_1APs in the paw oedema model. The endothelial cell PAR_1 target for thrombin may thus play a distinct role in the overall biological effects that thrombin can cause. The two selective PAR_1APs have previously been shown not to activate PAR_2 (Hollenberg *et al*., 1997; Vergnolle *et al*., 1998; Kawabata *et al*., 1999), and PAR_3 has been found to be refractory to PAR-AP activation (Ishihara *et al*., 1997). We cannot rule out the possibility, however, that these peptides activate either PAR_4 or another, as yet unidentified PAR, such as the proteinase-activated receptor in the rat jejunum which we found to be pharmacologically distinct from PAR_1, PAR_2 and PAR_3 (Vergnolle *et al*., 1998).

In conclusion, the results of the present study demonstrate that thrombin is able to exert both anti- and pro-inflammatory effects when injected into a rat hindpaw. In contrast to a previous report (Cirino *et al*., 1996), these studies suggest that the actions of thrombin cannot be attributed entirely to activation of PAR_1. Indeed, we provide evidence that the pro-inflammatory effects of thrombin are mechanistically distinct from the pro-inflammatory effects of selective PAR_1-activating peptides. Further studies are required to fully understand the mechanisms responsible for the pro- and anti-inflammatory activities of thrombin.

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References

BAR-SHAVIT, R., KAHN, A., MUDD, M.S., WILNER, G.D., MANN, K.G. & FENTON, J.W. (1984). Localization of a chemotactic domain in human thrombin. *Biochemistry,* **23,** 397–400.

BAR-SHAVIT, R., KAHN, A., WILNER, G.D. & FENTON, J.W. (1983). Monocyte chemotaxis: stimulation by specific exosite region in thrombin. *Science,* **220,** 728–731.

BARTOLI, F., LIN, H., GHOMASHCHI, F., GELB, M., JAIN, M., & APITZ-CASTRO, R. (1994). Tight binding inhibition of 85-kDa phospholipase A_2 but not 14-kDa phospholipase A_2 inhibit release of free arachidonate in thrombin-stimulated human platelets. *J. Biol. Chem.,* **269,** 15625–15630.

BILLS, T.K., SMITH J.B. & SILVER, M.J. (1977). Selective release of arachidonic acid from the phospholipids of human platelets in response to thrombin. *J. Clin. Invest.,* **60,** 1–6.

BIZIOS, R., LAI, L., FENTON, J.W. & MALIK, A.B. (1986). Thrombin-induced chemotaxis and aggregation of neutrophils. *J. Cell. Physiol.,* **128,** 485–490.

BLACKHART, B.D., EMILSSON, K., NGUYEN, D., TENG, W., MARELLI, A.J., NYSTED, T.S., SUNDELIN J. & SCARBOROUGH, R.M. (1996). Ligand cross-reactivity within the protease-activated receptor family. *J. Biol. Chem.,* **271,** 16466–16471.

CIRINO, G., CICALA, C., BUCCI, M.R., SORRENTINO, L., MARAGANORE, J.M. & STONE, S.R. (1996). Thrombin functions as an inflammatory mediator through activation of its receptor. *J. Exp. Med.,* **183,** 821–827.

DI ROSA, M., GIROUD, J.P. & WILLOUGHBY, D.A. (1971). Studies of the mediators of the acute inflammatory response induced in rats at different sites by carrageenan and turpentine. *J. Pathol.,* **104,** 15–29.

DRAIJER, R., ATSMA, D.E., VAN DER LAARSE, A. & HINSBERGH, V.W.M. (1995). cGMP and nitric oxide modulate thrombin-induced endothelial permeability: Regulation via different pathways in human aortic and umbilical vein endothelial cells. *Circ. Res.,* **76,** 199–208.

GLENN, K.C., FROST, G.H., BERGMANN, J.S. & CARNEY, D.H. (1988). Synthetic peptides bind to high-affinity thrombin receptors and modulate thrombin mitogenesis. *Peptide Res.,* **1,** 65–73.

HERBERT, J.M., DUPUY, E., LAPLACE, M.C., ZINI, J.M., BAR-SHAVIT, R. & TOBELEM, G. (1994). Thrombin induces endothelial cell growth via both a proteolytic and a non-proteolytic pathway. *Biochem. J.,* **303,** 227–231.

HOLLENBERG, M.D. (1996). Protease-mediated signalling: new paradigms for cell regulation and drug development. *Trends Pharmacol. Sci.,* **17,** 3–6.

HOLLENBERG, M.D., MOKASHI, S., LEBLOND, L. & DI MAIO, J. (1996). Synergistic actions of a thrombin-derived synthese peptide and a thrombin receptor-activating peptide in stimulating fibroblast mitogenesis. *J.Cell. Physiol.,* **169,** 491–496.

HOLLENBERG, M.D., SAIFEDDINE, M., AL-ANI, B. & KAWABATA, A.. (1997). Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides. *Can. J. Physiol. Pharmacol.,* **75,** 832–841.

HOLLENBERG, M.D., YANG, S.G., LANIYONU, A.A., MOORE, G.J. & SAIFEDDINE, M. (1992). Action of thrombin receptor polypeptide in gastric smooth muscle: identification of a core pentapeptide retaining full thrombin-mimetic intrinsic activity. *Mol. Pharmacol.,* **42,** 186–191.

ISHIHARA, H., CONNOLLY, A.J., ZENG, D., KAHN, M.L., ZHENG, Y.W., TIMMONS, C., TRAM, T. & COUGHLIN, S.R. (1997). Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature,* **386,** 502–506.

KAHN, M.L., ZHENG, Y.W., HUANG, W., BIGORNIA, V., ZENG, D., MOFF, S., FARESE Jr, R.V., TAM, C., COUGHLIN, S.R. (1998). A dual thrombin receptor system for platelet activation. *Nature,* **394,** 690–694.

KAWABATA, A., KURODA, R., MINAMI, T., KATAOKA, K. & TANEDA, M. (1998). Increased vascular permeability by a specific agonist of protease-activated receptor-2 in rat hindpaw. *Br. J. Pharmacol.,* **125,** 419–422.

KAWABATA, A., SAIFEDDINE, M., AL-ANI, B., LEBLOND, L. & HOLLENBERG, M.D. (1999). Evaluation of proteinase-activated receptor-1 (PAR-1) agonists and antagonists using a cultured cell receptor desensitization assay: activation of PAR-2 by PAR-1 targeted ligands. *J. Pharm. Exp. Ther.,* **288,** 358–370.

KRAMER, R., ROBERTS, E., MANETTA, J., HYSLOP, P. & JAKUBOWSKI, J. (1993). Thrombin-induced phosphorylation and activation of Ca^{2+}-sensitive cytosolic phospholipase A_2 in human platelets. *J. Biol. Chem.,* **268,** 26796–26804.

KRANZHOFER, R., CLINTON, S.K., ISHII, K, COUGHLIN, S.R., FENTON, J.W. & LIBBY, P. (1996). Thrombin potently stimulates cytokine production in human vascular smooth muscle cells but not in mononuclear phagocytes. *Circ. Res.,* **79,** 286–294.

MALIK, A.B. & FENTON, J.W. (1992). Thrombin-mediated increase in vascular endothelial permeability. *Sem. Thrombos. Hemostas.,* **18,** 193–199.

MARAGANORE, J.M., BOURDON, P., JABLONSKI, J., RAMACHANDRAN, K.L. & FENTON, J.W. (1990). Design and characterization of hirulogs: a novel class of bivalent peptide inhibitors of thrombin. *Biochemistry,* **29,** 7095–7101.

NELKIN, N.A., SOIFER, S.J., O'KEEFE, J., VU, T.K., CHARO, I.F. & COUGHLIN, S.R. (1992). Thrombin receptor expression in normal and atherosclerotic human arteries. *J. Clin. Invest.,* **90,** 1614–1621.

NYSTEDT, S., EMILSSON, K., WAHLESTEDT, C. & SUNDELIN, J. (1994). Molecular cloning of a potential proteinase activated receptor. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 9208–9212.

RASMUSSEN, U.B., VOURET-CRAVIARI, V., JALLAT, S., SCHLESINGER, Y., PAGES, G., PAVIRANI, A., LECOCQ, J.P., POUYSSEGUR, J. & VAN OBBERGHEN-SCHILLING, E. (1991). cDNA cloning and expression of a hamster alpha-thrombin receptor coupled to Ca^{2+} mobilization. *FEBS Lett.,* **288,** 123–128.

RAZIN, E. & MARX, G. (1984). Thrombin-induced degranulation of cultured bone marrow-derived mast cells. *J. Immunol.,* **133,** 3282–3285.

SKRZYPCZAK-JANKUN, E., CARPEROS, V.E., RAVICHANDRAN, K.G, TULINSKY, A., WESTBROOK, M. & MARAGANORE, J.M. (1991). Structure of the hirugen and hirulog 1 complexes of alpha-thrombin. *J. Mol. Biol.,* **221,** 1379–1393.

STANKOVA, J., ROLA-PLESZCZYNSKI, M.& D'ORLEANS-JUSTE, P. (1995). Endothelin 1 and thrombin synergistically stimulate IL-6 mRNA expression and protein production in human umbilical vein endothelial cells. *J. Cardiovasc. Pharmacol.,* **26,** S505–S507.

STIERNBERG, J., REDIN, W.R., WARNER, W.S. & CARNEY, D.H. (1993). The role of thrombin and thrombin receptor activating peptide (TRAP-508) in initiation of tissue repair. *Thromb. Haemostas.,* **70,** 158–162.

TAY-UYBOCO, J., POON, M.C., AHMAD, S. & HOLLENBERG, M.D. (1995). Contractile actions of thrombin receptor-derived polypeptides in human umbilical and placental vasculature: evidence for distinct receptor systems. *Br. J. Pharmacol.,* **115,** 569–578.

TOOTHILL, V.J., VAN MOURIK, J.A., NIEWENHUIS, H.K., METZELAAR, M.J. & PEARSON, J.D. (1990). Characterization of the enhanced adhesion of neutrophil leukocytes to thrombin-stimulated endothelial cells. *J. Immunol.,* **145,** 283–291.

UENO, A., MURAKAMI, K., YAMANOUCHI, K., WATANABE, M. & KONDO, T. (1996). Thrombin stimulates production of interleukin-8 in human umbilical vein endothelial cells. *Immunology,* **88,** 76–81.

VASSALLO, R.R., KIEBER-EMMONS, T., CICHOWSKI, K. & BRASS, L.F. (1992). Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J. Biol. Chem.,* **267,** 6081–6085.

VERGNOLLE, N., MACNAUGHTON, W.K., AL-ANI, B., SAIFEDDINE, M., WALLACE, J.L. & HOLLENBERG, M.D. (1998). Proteinase-activated receptor-2-activating peptides: identification of a receptor that regulates intestinal transport. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 7766–7771.

VU, T.K., HUNG, D.T., WHEATON, V.I. & COUGHLIN, S.R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell,* **64,** 1057–1068.

WEKSLER, B.B., LEY, C.W. & JAFFE, E.A. (1978). Stimulation of endothelial cell prostacyclin production by thrombin, trypsin, and the ionophore A23187. *J. Clin. Invest.,* **63,** 923–930.

XU, W., ANDERSEN, H., WHITMORE, T.E., PRESNELL, S.R., YEE, D.P., CHING, A., GILBERT, T., DAVIE, E.W. & FOSTER, D.C. (1998). Cloning and characterization of human protease-activated receptor 4. *Proc. Natl.Acad. Sci. U.S.A.,* **95,** 6642–6646.

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