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SPECIAL REPORT

Increased vascular permeability by a specific agonist of protease-activated receptor-2 in rat hindpaw

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The present study examined the effect of intraplantar (i.pl.) administration of a selective agonist of protease-activated receptor (PAR)-2, SLIGRL-NH_2(PP6-NH_2), on vascular permeability in rat hindpaw. PP6-NH_2, administered i.pl. at 10–100 nmol per paw, enhanced vascular permeability and caused oedema formation in rat hindpaw. SLIGRL (PP6-OH) and trypsin, by i.pl. administration, also elicited an increase in vascular permeability, although i.pl. administration of the mixture of constituent amino acids of PP6-OH at an equivalent dose did not. The PP6-NH_2-induced increase in vascular permeability was abolished by repeated pretreatment with compound 48/80 to deplete bioactive amines in mast cells. These findings suggest that the activation of PAR-2 induces acute inflammation, at least partially, *via* mast cell degranulation in rat hindpaw.

Keywords: Protease-activated receptor (PAR); protease; trypsin; thrombin; vascular permeability; inflammation; oedema; mast cell degranulation

Introduction Recently a new family of G-protein coupled, seven trans-membrane domain receptors has been described that are activated by proteolytic cleavage of their extracellular N-terminus (Vu *et al*., 1991; Nystedt *et al*., 1994; Bohm *et al*., 1996; Hollenberg, 1996; Ishihara *et al*., 1997). The first member of this family is the protease-activated receptor (PAR)-1/thrombin receptor. Thrombin cleaves the extracellular N-terminus of PAR-1, forming a new N-terminus (SFLLRN—, for human PAR-1), a 'tethered ligand', that interacts with some other region of the receptor (Vu *et al*., 1991; Hollenberg, 1996). The second member of this family is PAR-2, the activation of which occurs through the proteolytic unmasking of the receptor activating sequence (SLIGKV—, for human PAR-2; SLIGRL—, for murine PAR-2) by trypsin, but not by thrombin (Nystedt *et al*., 1994; Bohm *et al*., 1996; Hollenberg, 1996). Exogenously applied synthetic peptides as short as 5–6 amino acids based on the receptor activating sequence of PAR-1 and PAR-2 (for example, SFLLR and SLIGRL) can fully activate PAR-1 and PAR-2, respectively (Blackhart *et al*., 1996; Hollenberg *et al*, 1996; 1997; Kawabata *et al*., 1997).

Thrombin and/or PAR-1 (thrombin receptor) play a role in a variety of physiological and pathophysiological processes including inflammation. Thrombin causes mast cell degranulation (Razin & Marx, 1984), an increase in endothelial permeability (Malik & Fenton, 1992) and vasodilation (Muramatsu *et al*, 1992; Hollenberg *et al*., 1997) *in vitro*. Cirino *et al*. (1996) has demonstrated that thrombin acts as an inflammatory mediator *in vivo* through the activation of PAR-1, which in turn leads to release of vasoactive amines from mast cells. On the other hand, the physiological role of PAR-2 remains to be elucidated. The widespread distribution of PAR-2 in tissues not normally exposed to pancreatic trypsin indicates the existence of other physiological activators (Bohm *et al*., 1996). Interestingly, recent studies have identified tryptase as a potential activator of PAR-2 (Molino *et al*., 1997; Fox *et al*., 1997), which is a tetrameric trypsin-like enzyme present in mast cells and is released upon mast cell degranulation (Schwartz, 1994). In addition, it has been demonstrated that PAR-2 is induced by inflammatory mediators in human endothelial cells (Nystedt *et al*., 1996). Therefore, it is likely that PAR-2 plays a role in an inflammatory process. Here, we describe, for the first time, that a specific agonist of PAR-2, SLIGRL-NH_2 (PP6-NH_2), enhances *in vivo* vascular permeability in rat hindpaw.

Methods Male Wistar rats weighing 150–200 g (Japan SLC. Inc.) were anaesthetized by an intraperitoneal (i.p.) injection of urethane at 1.5 g kg^{-1}. Vascular permeability was measured as described previously (Cirino *et al*., 1996). Briefly, rats received an intravenous (i.v.) injection of 2.5% w/v Evans blue solution in 0.45% NaCl at a dose of 25 mg kg^{-1}, immediately before the intraplantar (i.pl.) injection of drugs or their vehicle in a volume of 100 μl. After 15 min, the rats were sacrificed by decapitation, and the i.pl. injected (right) and contralateral (left) hindpaws were removed and minced. The minced paws were incubated in 10 ml of formamide for 72 h at 37°C to extract Evans blue. Each solution was then filtered and the optical density of the filtrate was measured at 619 nm. The amount of Evans blue extravasated was determined by subtracting the Evans blue content in the contralateral paw from that in the drug-treated paw. In some experiments, the size of oedema was assessed by measuring the thickness of the hindpaw immediately before and 15 min after the i.pl. injection, and is expressed as % change from the value before i.pl. injection.

In the experiments to test involvement of the mast cell degranulation, the rats were depleted of their stores of histamine and 5-hydroxytryptamine by repeated injections of compound 48/80 as described by Di Rosa *et al*. (1971). Briefly, compound 48/80 (0.1% w/v in saline) was administered i.p. morning and evening for eight doses, starting with an evening

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dose. The dose employed was 0.6 mg kg^{-1} for the first six injections and 1.2 mg kg^{-1} for the last two injections. Control animals received i.pl. saline only in the same schedule. In preliminary experiments, the efficacy of the treatment was checked by confirming the absence of oedema for 1 h after i.pl carrageenin or the lack of the thrombin-induced increase in vascular permeability in the rats treated with compound 48/80. Vascular permeability in the depleted animals was measured as described above, except that the contralateral hindpaw received an i.pl. injection of saline in the same volume. Then, the difference between Evans blue contents in the drug-treated paw and in the saline-treated contralateral paw was defined as specific extravasation of Evans blue because the repeated treatment with compound 48/80 slightly affected the magnitude of the i.pl. saline-induced small increase in permeability.

All chemicals, trypsin from porcine pancreas, compound 48/80 (Sigma, U.S.A.), L-serine, L-leucine, L-isoleucine, L-glycine, L-arginine (Kishida, Japan), histamine hydrochloride (Wako, Japan), SLIGRL (Bachem, Switzerland; M.W., 657.8) and SLIGRL-NH_2 (Peptide Synthesis Core Facility, Department of Medical Biochemistry, University of Calgary, Canada; M.W., 656.8), were dissolved in physiological saline.

Results are expressed as the mean with s.e.mean. Statistical significance was analysed by Student's *t*-test or by Newman-Keul's test, and was set at a $P<0.05$ level.

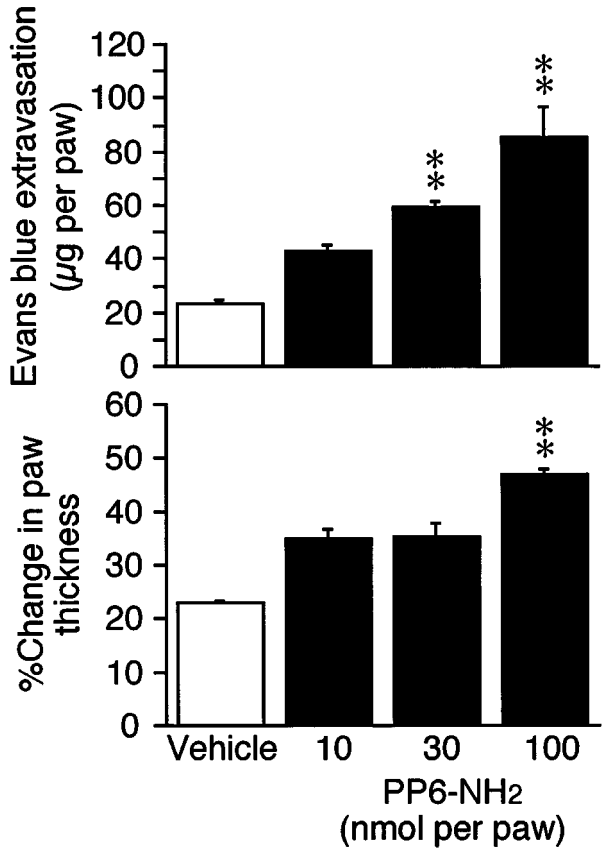

Figure 1 Effect of intraplantar (i.pl.) administration of SLIGRL-NH_2 (PP6-NH_2) on vascular permeability and paw thickness in the rat. The rat received an i.pl. injection of PP6-NH_2 at 10, 30 or 100 nmol per paw immediately after intravenous Evans blue at 25 mg kg^{-1}, and was killed after 15 min. Evans blue extravasated was determined by subtracting the Evans blue content in the untreated contralateral hindpaw from that in the drug-treated hindpaw. Paw thickness was measured immediately before and 15 min after PP6-NH_2. Data show the mean with s.e.mean from four to six rats. $^{**}P<0.01$ vs the vehicle-treated rats.

Results SLIGRL-NH_2 (PP6-NH_2), a specific agonist of PAR-2, when administered i.pl. at 10, 30 and 100 nmol per paw, markedly enhanced vascular permeability to Evans blue in a dose-dependent manner (Figure 1). The same dose range of PP6-NH_2 also caused a dose-dependent increase in the paw thickness as a parameter of oedema formation (Figure 1).

SLIGRL (PP6-OH) that is known to be less potent as a PAR-2 agonist than PP6-NH_2 (Hollenberg *et al*., 1996), administered i.pl. at 100 nmol per paw, also significantly increased Evans blue extravasation in the rat hindpaw, the magnitude of effect being smaller than that of PP6-NH_2 at the same dose. On the other hand, the amino acid cocktail solution consisting of 100 nmole of L-Ser, L-Ile, L-Gly and L-Arg and 200 nmole of L-Leu in 100 μl, which is equivalent to PP6-NH_2 at a dose of 100 nmol per paw in a volume of 100 μl, by i.pl. administration, did not enhance but slightly reduced vascular permeability in the hindpaw (Figure 2a). Trypsin administered i.pl. at 75 and 250 pmol per paw, but not at 25 pmol per paw,

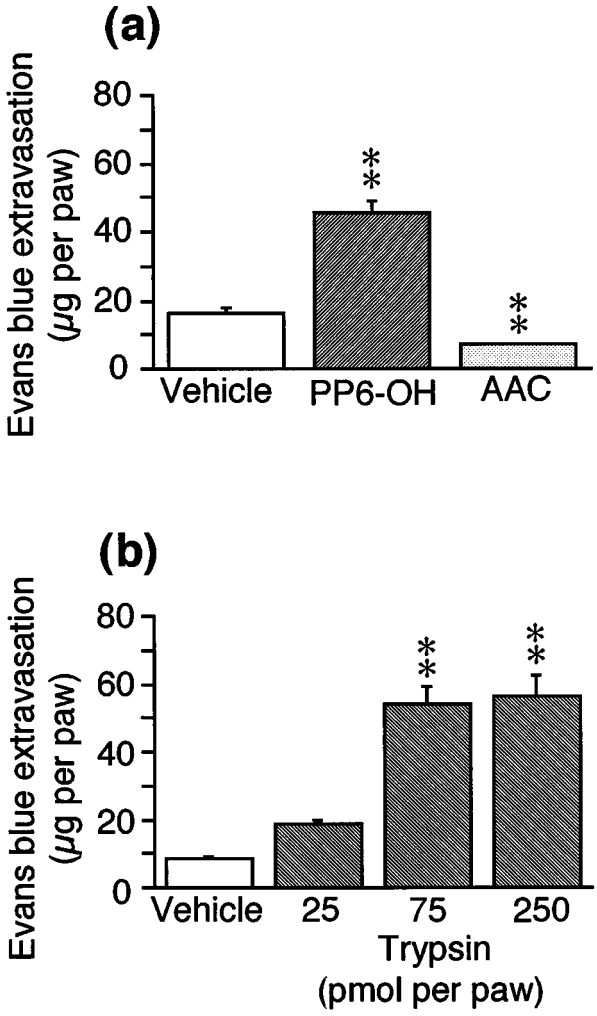

Figure 2 Effect of intraplantar (i.pl.) administration of SLIGRL (PP6-OH) (a) and trypsin (b) on vascular permeability in the rat. The rat received an i.pl injection of PP6-OH at 100 nmol per paw, an amino acid cocktail (AAC) consisting of L-Ser, L-Ile, L-Gly, L-Arg (each at 100 nmol per paw) and L-Leu (at 200 nmol per paw), or trypsin at 25, 75 and 250 pmol per paw, immediately after intravenous Evans blue at 25 mg kg^{-1}, and was killed after 15 min. Evans blue extravasated was determined by subtracting the Evans blue content in the untreated contralateral hindpaw from that in the drug-treated hindpaw. Data show the mean with s.e.mean from four rats. $^{**}P<0.01$ vs the vehicle-treated rats.

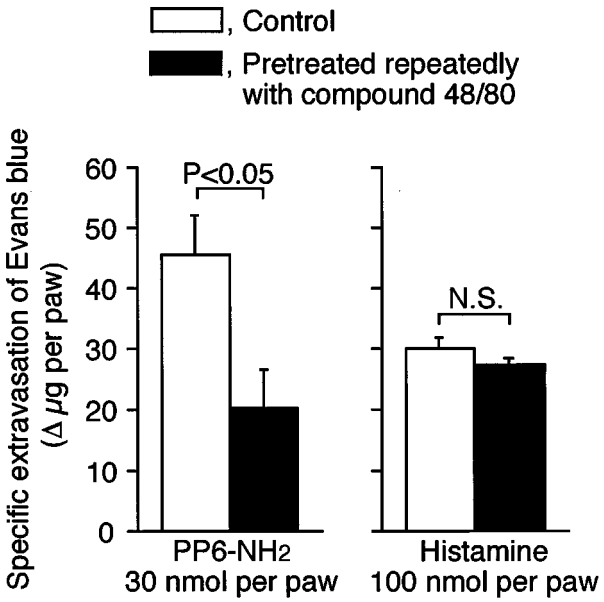

Figure 3 Effect of depletion of bioactive amines in mast cells on the PP6-NH_2-induced increase in vascular permeability in rat hindpaw. Rats were repeatedly treated with compound 48/80 to deplete bioactive amines in mast cells, and thereafter received i.pl. PP6-NH_2 at 30 nmol per paw or histamine at 100 nmol per paw in the left hindpaw and i.pl. saline in the contralateral hindpaw, immediately after intravenous Evans blue at 25 mg kg^{-1}, and was killed after 15 min. Specific extravasation of Evans blue was defined as the difference between Evans blue contents in the drug-treated paw and in the saline-treated contralateral paw. Data show the mean with s.e.mean from four rats. N.S., not significant.

significantly increased Evans blue extravasation in the hindpaw (Figure 2b).

In the rats previously depleted of their stores of histamine and 5-hydroxytryptamine, the PP6-NH_2-induced increase in vascular permeability was significantly reduced by 56%. In contrast, the effect of exogenous histamine, administered i.pl. at 100 nmol per paw, on vascular permeability was unaffected by repeated pretreatment with compound 48/80 (Figure 3).

Discussion The present study demonstrates, for the first time, that PP6-NH_2, a specific agonist of PAR-2, by i.pl. administration, enhances vascular permeability accompanied by formation of oedema in rat hindpaw, at least partially, *via* mast cell degranulation, strongly implying that PAR-2, as does PAR-1, plays a role in an inflammatory process.

PP6-NH_2, a synthetic peptide based on the receptor-activating sequence of murine PAR-2, is known to potently and selectively activate PAR-2 without activating PAR-1 at all, although peptides based on the receptor-activating sequence of mammalian PAR-1, such as SFLLR-NH_2, activate not only PAR-1 but also PAR-2 (Blackhart *et al*., 1996; Hollenberg *et al*., 1997; Kawabata *et al*., 1997). Therefore, the effect of PP6-NH_2 seen in the present study does not result from the activation of PAR-1. The finding that PP6-OH was less potent than PP6-NH_2 is consistent with the relative potency of PAR-2 agonists in the rat aorta relaxation assay system (Hollenberg *et al*., 1997). The lack of the effect of the constituent amino acid cocktail at a dose equivalent to 100 nmol per paw of PP6-OH also supports the specificity of the effect of the PAR-2 agonist. However, the possibility can not completely be excluded that PP6-NH_2 stimulated unknown receptors other than PAR-2, resulting in the increased permeability in the present study. Actually, a recent study (Roy *et al*., 1998) has suggested that PP6-NH_2 is capable of activating a receptor distinct from PAR-2, leading to an endothelium-dependent contraction in rat pulmonary artery. In future studies to reach final conclusion, therefore, the structure-activity relationship using various PAR-2 activating peptides and also inactive peptides, such as LSIGRL-NH_2, should be investigated in the *in vivo* permeability experiments. The development of a selective antagonist of PAR-2 would be beneficial to assure involvement of PAR-2 in an inflammation process.

The finding that trypsin, a PAR-2-activating enzyme, mimicked the effect of PP6-NH_2 or PP6-OH is in agreement with possible involvement of PAR-2 in inflammation. The relatively lower potency of trypsin seen in the present study may reflect poor diffusion of trypsin to the effective sites because of its large molecular size compared to PP6-NH_2. Subplantar regions of the hindpaw do not normally appear to be exposed to endogenous trypsin, suggesting the existence of unknown physiological activator enzymes. Recently, two candidate enzymes with favorable kinetic profiles that could cleave PAR-2 *in vivo*, namely acrosin from sperm and tryptase from mast cells were identified (Molino *et al*., 1997; Fox *et al*., 1997). Mast cell tryptase may be the most likely endogenous enzyme involved in the activation of PAR-2 related to inflammation, since tryptase is released upon mast cell degranulation (Schwartz, 1994).

The paw oedema caused by the activation of PAR-1 is exclusively attributable to mast cell degranulation (Cirino *et al*., 1996). Similarly, the PAR-2-mediated increase in vascular permeability appears to occur through mast cell degranulation, since the pretreatment with compound 48/80 to deplete histamine and 5-hydroxytryptamine in mast cells significantly abolished PP6-NH_2-induced Evans blue extravasation in rat hindpaw although it had no effect on the histamine-induced increase in vascular permeability. However, involvement of other mechanisms independent of mast cell degranulation also cannot be excluded considering that the effect of PP6-NH_2 did not completely disappear in spite of complete depletion of mast cell stores in the present study.

Taken together, the present study suggests a role of PAR-2 in inflammatory mechanisms.

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