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# Characterization of the inflammatory response to proteinase-activated receptor-2 ($PAR_2$)-activating peptides in the rat paw

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**1** In the present study, we have observed the development of an inflammatory reaction in the rat hindpaw, following the injection of specific agonists of $PAR_2$ (two $PAR_2$ activating peptides). This inflammation was characterized by oedema and granulocyte infiltration.

**2** Two selective $PAR_2$ activating peptides, SLGRL-$NH_2$ and *trans*-cinnamoyl-LIGRLO-$NH_2$ induced significant oedema in the rat hindpaw from 1–6 h following subplantar injection. Six hours after the $PAR_2$-activating peptide injection, the paw tissues showed a complete disruption of tissue architecture along with an inflammatory cell infiltrate.

**3** In the inflamed paw, $PAR_2$-immunoreactivity was expressed on endothelial cells as well as on the infiltrating inflammatory cells.

**4** The oedema induced by the injection of the two $PAR_2$ activating peptides was slightly reduced in rats pre-treated with compound 48/80, but was not modified by pre-treatment of rats with cromolyn, a mast cell stabilizer. Pre-treatment of rats with a cyclo-oxygenase inhibitor (indomethacin) or a nitric oxide synthase inhibitor (L-$N^{\omega}$-nitro-L-arginine methyl ester) had no effect on the oedema induced by the $PAR_2$-activating peptides.

**5** These results demonstrate that the administration of $PAR_2$-activating peptides into the rat paw induced an acute inflammatory response characterized by a persistent oedema (at least 6 h) and granulocyte infiltration. The $PAR_2$-induced inflammatory response occurred through a mechanism largely independent of mast cell activation, and of the production of prostanoids and nitric oxide.

**Keywords:** Proteinase-activated receptor; $PAR_2$; inflammation; oedema; mast cell; prostaglandin; nitric oxide

**Abbreviations:** L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester; LS-$NH_2$, LSIGRL-$NH_2$; $PAR_1$, proteinase-activated receptor-1; $PAR_2$, proteinase-activated receptor-2; $PAR_3$; proteinase-activated receptor-3; $PAR_4$, proteinase-activated receptor-4; PAR-APs, PAR-activating peptides; $PAR_2$APs, proteinase-activated receptor-2-activating peptides; PARs, proteolytically-activated-receptors; SL-$NH_2$, SLIGRL-$NH_2$; tc-$NH_2$, *trans*-cinnamoyl-LIGRLO-$NH_2$

## Introduction

$PAR_2$, is a member of a family of G protein-coupled receptors that are activated by proteinases (Proteinase-Activated Receptors: PARs) (Rasmussen *et al*., 1991; Vu *et al*., 1991; Nystedt *et al*., 1994; Ishihara *et al*., 1997; Kahn *et al*., 1998; Xu *et al*., 1998). Four members of this family have been cloned, three of which can be activated by thrombin ($PAR_1$, $PAR_3$ and $PAR_4$). The fourth, $PAR_2$, can be activated by trypsin or tryptase (Corvera *et al*., 1997; Molino *et al*., 1997). The unique mechanism whereby proteinases activate the PARs involves the proteolytic cleavage and unmasking of an N-terminal receptor sequence, that in turn acts as a tethered ligand, which activates the receptor, itself. Short synthetic peptides based on the proteolytically revealed receptor sequences (PAR-activating peptides: PAR-APs) can, in isolation, selectively activate $PAR_1$ (e.g., the peptide TFLLR-$NH_2$) and $PAR_2$ (e.g. SLIGRL-$NH_2$: SL-$NH_2$). $PAR_3$ and $PAR_4$ are both activated by thrombin, but they do not appear to be activated by the PAR-APs derived from $PAR_1$ and $PAR_2$.

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The physiological and pathophysiological importance of $PAR_2$ is largely unknown. Previous studies have shown that $PAR_2$ activation can cause relaxation of rat aorta rings by an endothelium-dependent mechanism mediated by nitric oxide production (Al-Ani *et al*., 1995; Saifeddine *et al*., 1996). In the intact rat, the intravenous injection of a $PAR_2$-activating peptide has been observed to produce a marked fall in blood pressure (Hwa *et al*., 1996; Emilsson *et al*., 1997). The possible involvement of $PAR_2$ in the inflammatory response has been suggested by the finding that $PAR_2$ mRNA is upregulated in cultured endothelial cells by interleukin-1$\alpha$ and tumour necrosis factor-$\alpha$ (Nystedt *et al*., 1996). Moreover, mast cell tryptase can cleave and activate $PAR_2$ *in vitro* in endothelial cells, enterocytes and colonic myocytes (Molino *et al*., 1997; Corvera *et al*., 1997; Fox *et al*., 1997).

The studies summarized in the above paragraph suggested that $PAR_2$ may play an important role in inflammation. A recent study has shown that the injection into the rat paw of the $PAR_2$-AP, SL-$NH_2$, can enhance vascular permeability and cause swelling (Kawabata *et al*., 1998). This vascular effect was observed at a single time point (15 min) after the peptide was administered. Unfortunately, no other components of an inflammatory response were examined. By using the inflammatory model

of rat hindpaw oedema (Winter *et al*., 1962), we wished to determine if $PAR_2$ activation would result in an inflammatory reaction that included infiltration of granulocytes, and we wished to examine in further detail, the time course and the mechanism of this inflammatory response (i.e., involvement of mediators such as nitric oxide and prostaglandins). Two selective $PAR_2$APs were injected into the paw of rats: the SL-$NH_2$ peptide, which corresponds to the tethered-ligand sequence of murine and rat $PAR_2$, and *trans*-cinnamoyl-LIGRLO-$NH_2$ (tc-$NH_2$), a peptide which has been found to be highly selective for $PAR_2$ (Vergnolle *et al*., 1998; Saifeddine *et al*., 1998) and which is more resistant to metabolic degradation by aminopeptidases, than SL-$NH_2$. A partial reverse sequence peptide of the $PAR_2$AP, LSIGRL-$NH_2$, which is unable to activate $PAR_2$ (Hollenberg *et al*., 1997), was used as a control agonist for paw injection. In addition, we examined the role of mast cells, nitric oxide and prostanoids in mediating the effects of $PAR_2$ activation.

## Methods

### *Animals*

Male, Wistar rats (175–200 g) obtained from Charles River Breeding Farms (Montreal, QC, Canada) were used. Animals 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*

The animals were lightly anaesthetized with halothane (5%). Basal paw volume was recorded using a hydroplethismometer (Ugo Basile, Milan, Italy). A subplantar injection of 0.1 ml of the test substance was then administered ($n=6$ per group in all experiments). The peptides were initially dissolved as stock solutions (1–5 mM) in 25 mM HEPES (pH 7.4) and were diluted for injection in sterile 0.9% saline to give the desired concentrations. The test substances included the $PAR_2$APs, SL-$NH_2$ and tc-$NH_2$ (100 and 500 $\mu$g) and the control peptide LS-$NH_2$ (100 and 500 $\mu$g) which is not able to activate $PAR_2$. Paw volume was measured every hour for 6 h after the peptide injection. The increase in paw volume was evaluated as difference between the paw volume at each interval and the basal paw volume.

For the evaluation of the effects of drugs on oedema formation, some groups of rats were pre-treated with indomethacin (5 mg $kg^{-1}$ given orally) or L-$N^{\omega}$-nitro-L-arginine methyl ester (L-NAME; 25 mg $kg^{-1}$ in saline, intraperitoneally) 1 h before the subplantar injection of tc-$NH_2$. The control group for indomethacin received the appropriate vehicle (5% $NAHCO_3$) and the control group for L-NAME received intraperitoneally, D-NAME, 25 mg $kg^{-1}$ in saline. Other groups of rats were treated with compound 48/80 to deplete mast cells, 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. The control groups received a saline injection (the vehicle for 48/80). Other groups of rats were treated with sodium cromoglycate (cromolyn), a mast cell stabilizer, at 20 mg $kg^{-1}$ by intravenous injection 1 h before the subplantar injection of the peptides SL-$NH_2$ and tc-$NH_2$ and again 1 h after the subplantar injection of SL-$NH_2$ and tc-$NH_2$. The control group received a saline injection (the vehicle for cromolyn) at the same time-points.

### *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 (1–5 mM) prepared in 25 mM HEPES buffer, pH 7.4, were analysed by quantitative amino acid analysis to verify peptide concentration and purity. Indomethacin, L-NAME, compound 48/80 and cromolyn were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.).

### *Histology*

Rats were given a 0.1 ml subplantar injection of either the inactive peptide LS-$NH_2$, one of the $PAR_2$APs (SL-$NH_2$ and tc-$NH_2$), or vehicle ($n=3$ per group) and were killed 6 h thereafter. The injected paws were removed and fixed by immersion in formalin. The paws were then washed in 50% ethanol in distilled water an embedded in paraffin wax. Sections (5 $\mu$m) were mounted on glass slides and stained with haematoxylin and eosin.

### *Histochemistry*

Rats were given a 0.1 ml subplantar injection of either the inactive peptide LS-$NH_2$, the $PAR_2$AP SL-$NH_2$, or vehicle ($n=3$ per group) and were killed 6 h thereafter. The injected paws were removed and fixed by immersion in Carnoy's fixative (ethanol/chloroform/acitic acid, 6 : 3 : 1). The paws were then washed in 50% ethanol in distilled water and embedded in paraffin wax. Sections (5 $\mu$m) were mounted on glass slides and stained with Alcian blue 1% (a mast cell-specific dye) and counterstained with Safranin 1%.

### *Immunohistochemistry*

Rats were given a 0.1 ml subplantar injection of either the control peptide LS-$NH_2$ or the $PAR_2$AP tc-$NH_2$ and were killed 6 h after. The injected paws were removed and fixed by immersion in Zamboni's fixative overnight at 4°C. After fixation, they were washed in a phosphate buffered saline (pH 7.4) and cryostat sections (12 $\mu$m) were processed for immunohistochemistry. Briefly, sections were incubated for 48 h at 4°C in a specific anti-$PAR_2$ antibody raised in rabbit (B5, 1 : 1000; Kong *et al*., 1997). After washing, they were incubated in a donkey anti-rabbit IgG conjugated to CY3 (1 : 100, Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) secondary antibody for 1 h at room temperature. Finally they were washed and mounted in bicarbonate-buffered glycerol (1 : 3, pH 8.6). Controls were treated as above, but incubated in primary antibody containing 10 $\mu$g $ml^{-1}$ of the original polypeptide hapten

used to raise the antibody. Sections were viewed under a Zeiss Axioplan fluorescence microscope. Micrographs were made by digital capture of the images using a Sony CCD camera and Northern Exposure (Empix Inc., ON, Canada) software. Montages of the original images were made with Corel Draw.

### *Statistical analysis*

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

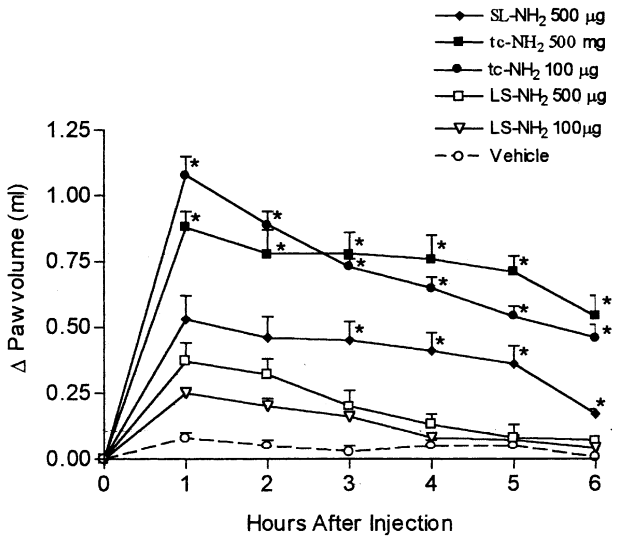


**Figure 1** Increase in rat hindpaw volume following administration of PAR$_2$-activating peptides (SL-NH$_2$, 500 $\mu$g and tc-NH$_2$, 100 and 500 $\mu$g), a control peptide (LS-NH$_2$, 100 and 500 $\mu$g) or vehicle. Asterisks denote significant differences ($P<0.05$) between the control peptide groups and the other groups for the same dose.

## Results

### *Oedema induced by PAR$_2$ activation*

Subplantar injection of the PAR$_2$APs (SL-NH$_2$ and tc-NH$_2$) or of an inactive peptide (LS-NH$_2$) caused oedema in the first 2 h, the magnitude of which was significantly greater than that caused by the vehicle alone. Nonetheless, from the third to the sixth hour after the injection, the oedema elicited by 500 $\mu$g (765 nmoles) of the selective PAR$_2$-activating peptide SL-NH$_2$ was significantly greater than that caused by the same dose of the control peptide (LS-NH$_2$) that is unable to activate PAR$_2$ (Figure 1). Injection of either 100 or 500 $\mu$g (125 or 615 nmoles) of another selective PAR$_2$AP (tc-NH$_2$), which is more resistant to metabolic degradation by aminopeptidases than SL-NH$_2$, provoked a profound oedema, significantly greater than the response caused by the inactive peptide (LS-NH$_2$), at all time points (Figure 1).

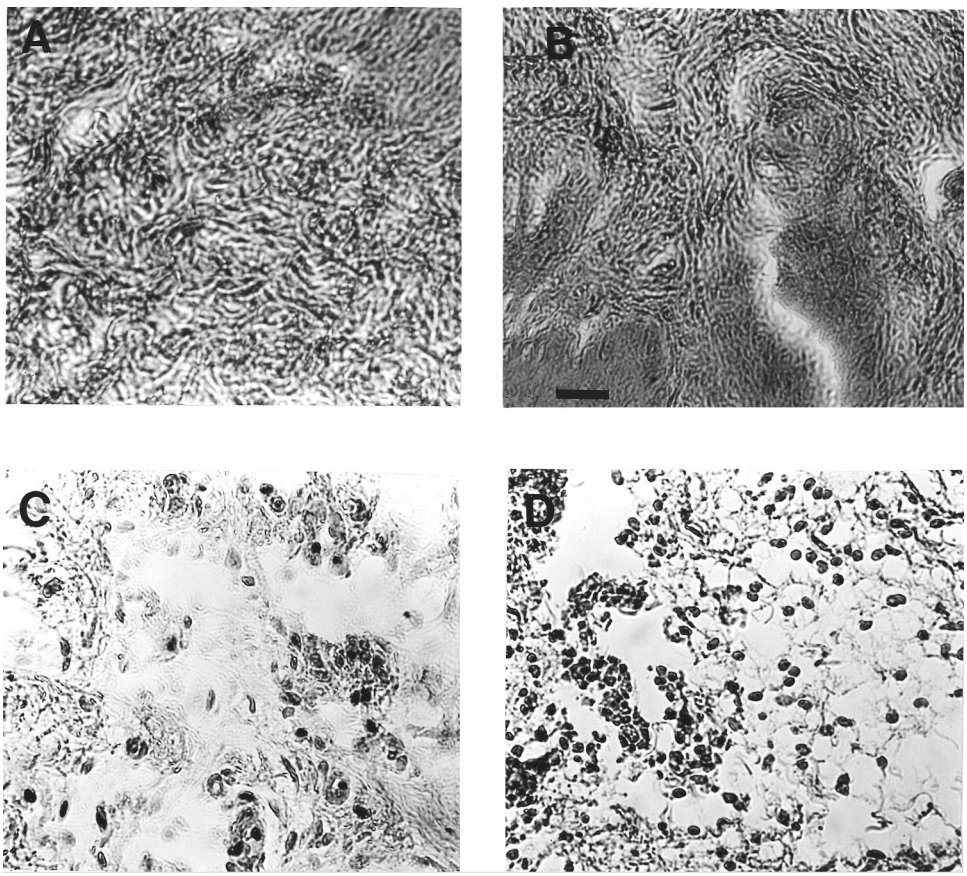


**Figure 2** Histological staining of sections of rat hindpaws, 6 h after the injection of vehicle (A), the control peptide (LS-NH$_2$, 100 $\mu$g) (B), the two PAR$_2$-activating peptides SL-NH$_2$, 500 $\mu$g (C), and tc-NH$_2$, 100 $\mu$g (D). Scale bar: 50 $\mu$m. The scale bar in B is for A, B, C and D.

*Histological alterations associated with $PAR_2$ activation*

In rats injected with saline, no sign of tissue damage or cellular infiltration was observed (Figure 2A). The paws of rats injected with the control peptide, LS-$NH_2$, exhibited some disruption of tissue architecture, but only in restricted areas. Moreover, there was a very low degree of cell infiltration (Figure 2B). In contrast, paws injected with either of the $PAR_2$APs (SL-$NH_2$ and tc-$NH_2$) exhibited profound oedema and complete disruption of tissue architecture (Figure 2C and D). Numerous inflammatory cells (mainly granulocytes) were evident in the paws of rats injected with the $PAR_2$APs.

*Localization of $PAR_2$ receptors*

The localization of the $PAR_2$ receptors was assessed by immunohistochemistry. $PAR_2$-immunoreactivity was observed at very low levels in the contralateral (non-injected) paw of rats treated with the $PAR_2$AP or in the injected paw of rats treated with the control peptide (LS-$NH_2$). In the animals injected with the control peptide (LS-$NH_2$), there was faint staining of the endothelium of blood vessels and in a few resident cells in the sub-dermis (Figure 3). Muscle and connective tissue were not labelled. In animals injected 6 h previously with the $PAR_2$-activating peptide, tc-$NH_2$, there was extensive oedema and cellular infiltration. $PAR_2$-immunoreactivity was found on infiltrating cells and was up-regulated on the endothelium of some blood vessels (mostly venules, Figure 3). Incubation of sections with antibody containing the hapten resulted in the complete abolition of staining, suggesting that the labelling observed was specific to $PAR_2$. However, since other protein epitopes might conceivably cross-react with our anti-$PAR_2$ antiserum, we refer to the labelling as $PAR_2$-immunoreactivity.

*Role of mast cells*

Administration of the control peptide (LS-$NH_2$) to rats that had been pretreated with compound 48/80 or with cromolyn resulted in a paw oedema response that was not significantly different from that observed in response to saline injection (Figure 4A and B). Pre-treatment with compound 48/80 resulted in a small reduction of the oedema response to the two selective $PAR_2$APs, from the second to the sixth hour after the injection of tc-$NH_2$ and from the fourth to the fifth hour after the injection of the SL-$NH_2$ peptide (Figure 4A). The pre-treatment of animals with doses of cromolyn known to inhibit hypersensitivity reaction in the rat hindpaw (Martel & Klicius, 1997), had no effect on the oedema induced by either tc-$NH_2$ or SL-$NH_2$ (Figure 4B). The histochemical study showed that some mast cells stained positively with Alcian blue in the paws

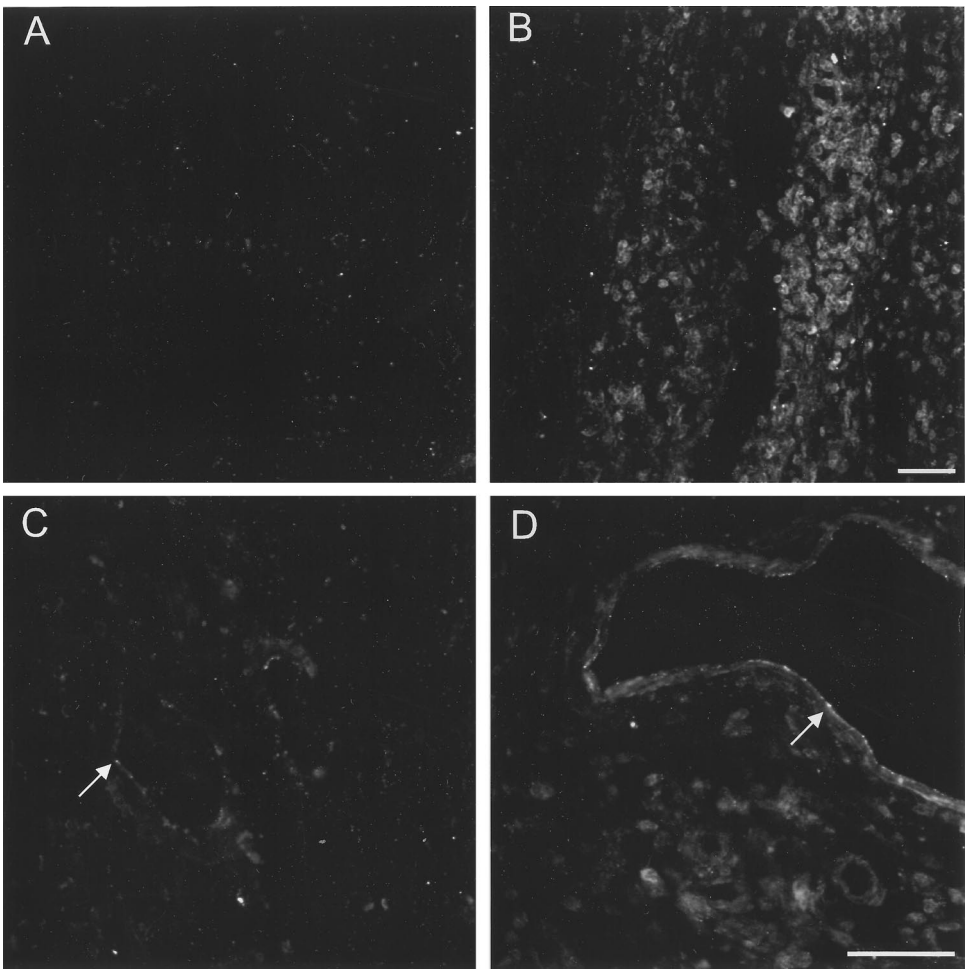


**Figure 3** Fluorescence micrographs of $PAR_2$-immunoreactivity in the rat paw from animals injected 6 h earlier with a control peptide (LS-$NH_2$, A and C) and paws injected with a $PAR_2$AP (tc-$NH_2$, B and D). (A) Subdermis from the control paw showing little or no specific labelling. (B) In paws injected with the $PAR_2$AP, there was an extensive infiltration of immunoreactive cells and considerable oedema. (C) On the endothelium of some blood vessels of control tissues (arrow) there was faint $PAR_2$-immunoreactivity. (D) In $PAR_2$AP-treated paws, increased intensity of $PAR_2$-immunoreactivity was found on the endothelium of some blood vessels (mostly venules, arrow). Scale bars: 50 $\mu$m. The scale bar in B is for A and B, and the bar in D is for C and D.

injected with buffer. In the paws injected with the inactive peptide LS-$NH_2$, the Alcian blue staining was diffuse around some mast cells indicating a degranulation. In that tissues, the most part of mast cells failed to take up Alcian blue because of the release of their granule contents. In paws injected with the selective $PAR_2AP$ SL-$NH_2$, several mast cells stained positively with Alcian blue among the numerous infiltrated inflammatory cells. A higher amount of Alcian blue-stained mast cells was observed in SL-$NH_2$-injected paws compared to paws injected with buffer.

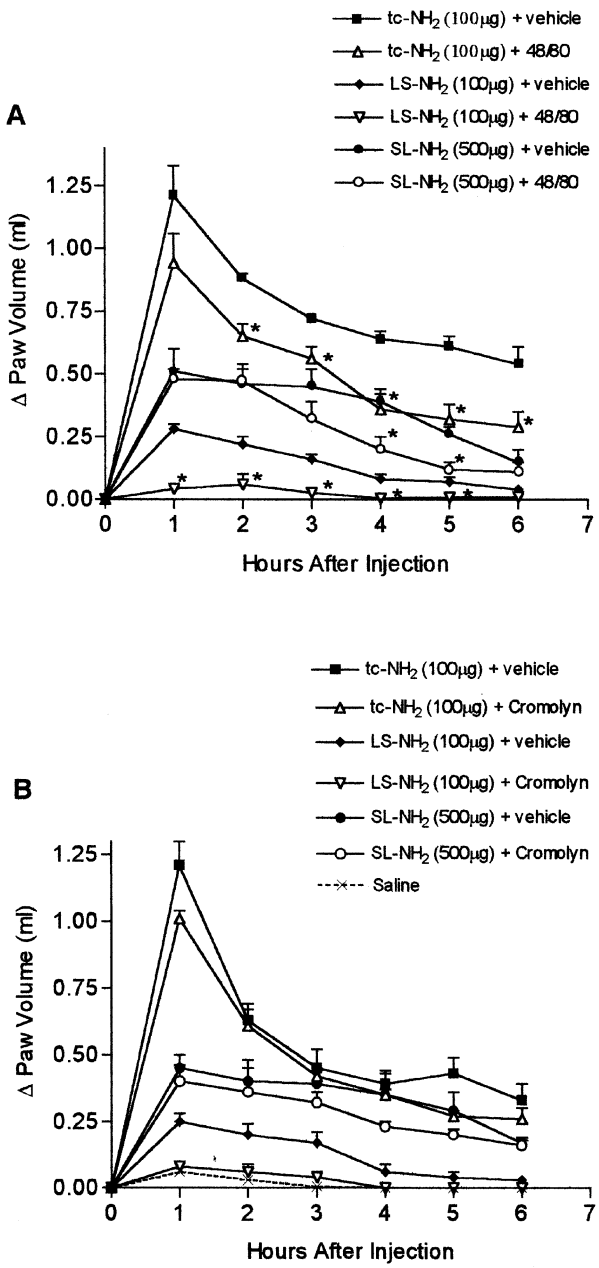


**Figure 4** (A) Effects of prior depletion of mast cells (through pre-treatment with compound 48/80) on the increase in hindpaw volume following injection of $PAR_2$-activating peptides (tc-$NH_2$, 100 $\mu$g or SL-$NH_2$ 500 $\mu$g) or a control peptide (LS-$NH_2$, 100 $\mu$g). Asterisks denote significant differences ($P<0.05$) from the corresponding group not treated with compound 48/80. (B) Effects of mast cell stabilization (through pre-treatment with cromolyn) on the increase in hindpaw volume following injection of $PAR_2$-activating peptides (tc-$NH_2$, 100 $\mu$g or SL-$NH_2$ 500 $\mu$g) or a control peptide (LS-$NH_2$, 100 $\mu$g). Asterisks denote significant differences ($P<0.05$) from the corresponding group not treated with cromolyn.

### *Role of prostanoids*

Pre-treatment of the rats with indomethacin at a dose known to inhibit carrageenan-airpouch inflammation (Wallace *et al.*, 1999) resulted in a small but significant decrease in the oedema induced by the $PAR_2AP$, tc-$NH_2$, but this decrease was not apparent until the third through sixth hours after administration of tc-$NH_2$. Indomethacin had no effect on the oedema induced by the control peptide, LS-$NH_2$ (Figure 5).

### *Role of nitric oxide*

Pre-treatment of the rats with 25 mg $kg^{-1}$ of L-NAME, a dose that has been previously shown to significantly inhibit the carrageenan-induced paw oedema (Honore *et al.*, 1995), did not significantly affect the oedema response to either the $PAR_2AP$, tc-$NH_2$, or the control peptide, LS-$NH_2$ (Figure 6).

## Discussion

In comparison to $PAR_1$, little is known about the physiological and pathophysiological importance of $PAR_2$. Some studies have shown that $PAR_2$ activation leads to effects characteristic of an inflammatory response, such as blood vessel relaxation (Al-Ani *et al.*, 1995; Saifeddine *et al.*, 1996), an increase in vascular permeability (Kawabata *et al.*, 1998) and hypotension (Hwa *et al.*, 1996; Emilsson *et al.*, 1997). However, the ability of $PAR_2$ activation to play a role in other aspects of the inflammatory response, such as leukocyte margination, granulocyte infiltration, cytokine production, prolonged oedema and a disruption of tissue integrity, has yet to be explored in any depth. The study we describe here considerably extends that of Kawabata and colleagues (1998) who demonstrated a rapid (15 min) mast cell-dependent increase in tissue permeability (Evan's blue extravasation) caused by the administration of the $PAR_2AP$, SL-$NH_2$. In our study, we have shown that an acute inflammatory response characterized by persistent oedema (at least 6 h), a dramatic alteration of

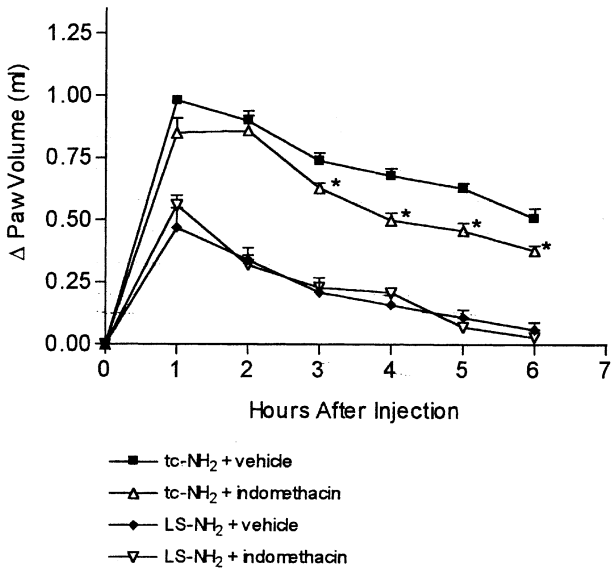


**Figure 5** Effects of pre-treatment with the cyclo-oxygenase inhibitor, indomethacin, on the increase in hindpaw volume following injection of $PAR_2$-activating peptide (tc-$NH_2$, 100 $\mu$g) or a control peptide (LS-$NH_2$, 100 $\mu$g). Asterisks denote significant differences ($P<0.05$) from the corresponding group not treated with indomethacin.

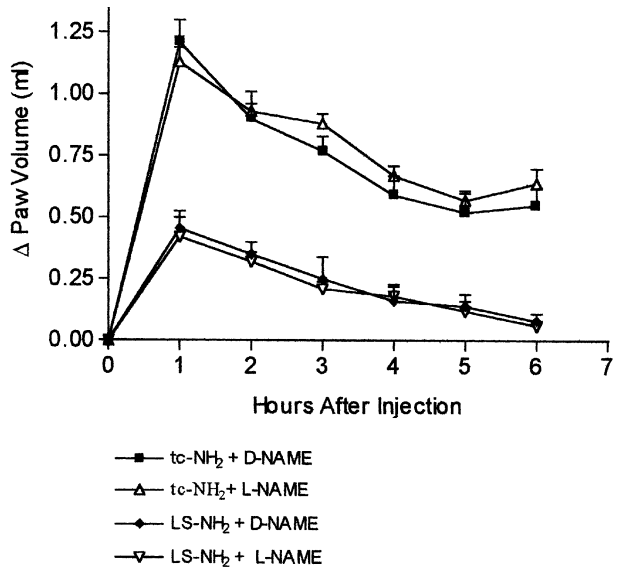


**Figure 6** Effects of pre-treatment with the nitric oxide synthase inhibitor, L-NAME, on the increase in hindpaw volume following injection of a PAR$_2$-activating peptide (tc-NH$_2$, 100 $\mu$g) or a control peptide (LS-NH$_2$, 100 $\mu$g), compared to control treatment with D-NAME. Asterisks denote significant differences ($P<0.05$) from the corresponding group not treated with L-NAME.

tissue cyto-architecture and robust granulocyte infiltration was induced by two selective PAR$_2$APs, tc-NH$_2$ and SL-NH$_2$. Moreover, we have shown that the rapid mast cell-dependent increase in vascular permeability observed by Kawabata *et al.* (1998) may be due only to a minor extent to a PAR$_2$-mediated process, since the PAR$_2$-inactive peptide LS-NH$_2$ can also cause a mast cell-dependent oedema response. Finally, we demonstrated that the acute inflammatory response induced by the potent and selective PAR$_2$AP, tc-NH$_2$, is largely independent of mast cell degranulation and does not involve either prostaglandins or nitric oxide. These findings strongly suggest that PAR$_2$, present in tissue components other than mast cells, can play a pivotal role in the inflammatory process.

In the present study, the magnitude of the oedema response provoked by PAR$_2$ activation after the injection of tc-NH$_2$ was as great as that observed following the injection of 1% (wt vol$^{-1}$) carrageenan in the well-documented model of rat hindpaw oedema (Winter *et al.*, 1962). Moreover, PAR$_2$ activation in the rat paw resulted not only in an increase in vascular permeability, as reflected by the oedema, but also in the marked infiltration of granulocytes, another hallmark feature of inflammatory reactions. Endothelial cells, which express PAR$_2$ (Al-Ani *et al.*, 1995; Hwa *et al.*, 1996; Mirza *et al.*, 1996; Saifeddine *et al.*, 1996), may represent one of the principal targets for PAR$_2$APs in the paw, in terms of causing increased tissue permeability and oedema. In accord with previous work documenting the presence of PAR$_2$ on neutrophils (Howells *et al.*, 1997), we found that in tissues of rats that had been injected with the PAR$_2$AP, tc-NH$_2$, PAR$_2$ was expressed on infiltrating inflammatory cells as well as on endothelial cells. These results suggest that in addition to its effects on endothelial cells, PAR$_2$ may also have an impact on the inflammatory reaction *via* its ability to activate cells that migrate into the tissue. In this regard, it is important to note that for at least 6 h after the injection of the PAR$_2$APs a prominent inflammatory reaction was still present, both in terms of oedema and disruption of tissue cyto-architecture.

Surprisingly, we found that a significant oedema response (compared to that observed with saline alone) could be detected during the first 3 h following administration of a control peptide, LS-NH$_2$, that cannot activate PAR$_2$. Comparable results were observed in a previous study we performed with the same model of inflammation but using peptides that selectively activate PAR$_1$ rather than PAR$_2$ (Vergnolle *et al.*, 1999). In that study, the PAR$_1$ control peptide FSLLR-NH$_2$, which cannot activate PAR$_1$, caused an oedema significantly greater than that observed with saline alone, but less than the oedema caused by a PAR$_1$-selective peptide agonist. Like the results we have described for the control PAR$_1$ peptide FSLLR-NH$_2$ (Vergnolle *et al.*, 1999), the response to the control PAR$_2$ peptide LS-NH$_2$ (a partial reverse-sequence peptide of the PAR$_2$ peptide) was completely abolished by pre-treating the animals with compound 48/80 or cromolyn. Moreover, in the present study, the histochemical observations showed that the injection of the LS-NH$_2$ peptide caused mast cell degranulation. Therefore, the effect on the oedema response observed with these control peptides that can not activate PAR$_1$ or PAR$_2$, appeared to be due to mast cell activation *via* a mechanism that does not involve either PAR$_1$ or PAR$_2$. It is possible that this mast cell-dependent oedema response to control peptides may have been attributable to the aromatic/basic residues in these peptides. Such residues could potentially activate the mast cell in a manner similar to the activation caused by compound 48/80, a formaldehyde Schiff-base conjugate that can also expose mast cells to both aromatic and basic substituents (Mousli *et al.*, 1990). We cannot exclude the possibility that the PAR$_1$ and PAR$_2$ control peptides might also activate receptors other than PAR$_1$ or PAR$_2$ that are located on mast cells to cause a mast cell-dependent oedema.

The mechanism of action of the selective PAR$_2$APs in terms of inducing oedema formation is not clear. As mentioned above, a recent report (Kawabata *et al.*, 1998) described an increase in vascular permeability induced by the intraplantar injection of the SL-NH$_2$ peptide that was eliminated by treating the animals with compound 48/80. Our observations, with respect to oedema formation in rats treated with compound 48/80, suggest a more limited role for the mast cell component than that was suggested by the study of Kawabata *et al.* (1998) which focused primarily on changes in tissue permeability. Our work showed that in rats pre-treated with compound 48/80, the oedema induced by the injection of the PAR$_2$APs was only slightly reduced from the second to the sixth hour for the tc-NH$_2$ peptide and from the forth to the fifth hour for the SL-NH$_2$ peptide. Even at 6 h after the tc-NH$_2$ injection, the oedema in compound 48/80-treated animals was not abolished and was still greater than in animals injected either with the control peptide or saline. In contrast, the oedema induced by the control PAR$_2$ peptide, LS-NH$_2$, was completely abolished by pre-treatment of the rats with compound 48/80. These differences between the results of Kawabata *et al.* (1998) and ours may be explained primarily by differences in the time frame of the two studies. In the present study, the oedema responses were monitored for 6 h following the injection of the PAR$_2$AP, while in the study of Kawabata *et al.* (1998) the vascular permeability was measured at only one time-point: 15 min after peptide injection. At such an early time point after peptide injection (15 min), it may be difficult to distinguish between the events mediated by PAR$_2$ activation and the processes triggered either by LS-NH$_2$ or SL-NH$_2$ in mast cells *via* a receptor other than PAR$_2$. Another factor to explain the differences between our observations and those reported by Kawabata *et al.* (1998) may be related to the different peptides and the higher doses we have used. In our

study, we have used doses of 100 and 500 $\mu$g (125–615 nmoles for the tc-NH$_2$; 155–765 nmoles for the SL-NH$_2$ peptide) of two PAR$_2$APs, tc-NH$_2$ and SL-NH$_2$, and of the control LS-NH$_2$ peptide. The same doses of peptide were used in a previous study that demonstrated the pro-inflammatory effects of a PAR$_1$-activating peptide in the rat hindpaw model (Cirino *et al*., 1996). Because of the non-PAR$_2$-mediated effect of the control peptide (LS-NH$_2$) on the oedema response, we were unable to detect with confidence, differences between the control peptide and the PAR$_2$-activating peptide (SL-NH$_2$) at doses below 100 $\mu$g (155 nmoles; data not shown). In the study of Kawabata *et al*. (1998), the highest dose of SL-NH$_2$ they used was 65 $\mu$g (100 nmoles), and a mixture of amino acids rather than an intact scrambled sequence peptide was used as a 'control'. Thus, the mast cell-dependent effects of low doses of SL-NH$_2$ ($\leqslant$65 $\mu$g or 100 nmoles) on vascular permeability that were described by Kawabata *et al*. (1998) may have been due not only to the activation of PAR$_2$ on mast cells, but also to the activation of another mast cell receptor. In our study, the use of higher doses of the SL-NH$_2$ peptide (500 $\mu$g; 765 nmoles) or the use of the more potent tc-NH$_2$ peptide, which is more resistant to aminopeptidase degradation than SL-NH$_2$, allowed us to distinguish the effect of PAR$_2$ activation from the mast cell-dependent effect induced most likely *via* a receptor other than PAR$_2$ or by a non-receptor mediated mechanism (Mousli *et al*., 1990). Apart from the compound 48/80 treatment, we have also treated two groups of rats with cromolyn, a mast cell stabilizer, and observed that the oedema induced by the injection of each of the PAR$_2$APs was not modified (Figure 4B). The experiments performed with 48/80- and cromolyn-treated animals clearly showed that the oedema induced by the injection of PAR$_2$APs is largely unrelated to mast cell activation. Nonetheless, histochemical examination indicated that among the numerous infiltrated inflammatory cells observed in paws 6 h after the injection of the PAR$_2$AP SL-NH$_2$, several were identified as mast cells. These results indicate that even if the PAR$_2$APs-induced oedema is largely independent of mass cell activation, mast cells may participate in a later phase of the inflammatory reaction initiated by PAR$_2$ activation. PAR$_2$ activation has been shown to result in the release of arachidonic acid and the generation of prostaglandin E$_2$ and F$_{1\alpha}$ (Kong *et al*., 1997). These mediators could contribute to the inflammatory response. However, the oedema caused by the injection of the PAR$_2$AP, tc-NH$_2$, was inhibited only to a small extent by indomethacin, suggesting that the pro-inflammatory effect was mostly independent of prostanoids. PAR$_2$ activation can also result in the liberation of nitric oxide (Al-Ani *et al*., 1995; Hwa *et al*., 1996; Saifeddine *et al*., 1996). However, we found that pre-treatment with a nitric oxide synthase inhibitor (L-NAME) failed to modify the oedema response to a selective PAR$_2$AP (tc-NH$_2$).

PAR$_2$ can be activated by trypsin in certain tissues, such as enterocytes (Kong *et al*., 1997). However, other tissues that express PAR$_2$ are not exposed to trypsin. Other proteinases might therefore be responsible for PAR$_2$ activation *in vivo*, particularly in the case of inflammatory reactions. Mast cells are involved in many inflammatory reactions as effector cells that initiate the inflammatory response by releasing a variety of pro-inflammatory mediators. Among the mediators released during mast cell degranulation, proteinases represent the major protein constituents. Tryptase, which is one of the proteinases released by activated human mast cells, is able to cleave and activate PAR$_2$ (Corvera *et al*., 1997; Fox *et al*., 1997; Molino *et al*., 1997). Thus, tryptase or other mast cell proteinases represent good candidates for the activation of PAR$_2$ *in vivo* during inflammatory processes. PAR$_2$ activation by mast cell proteinases may play an important role in inflammatory states characterized by mast cell infiltration and degranulation. To date, the use of peptides that selectively activate PAR$_2$ has provided considerable insights concerning the possible consequences of activating PAR$_2$ *in vivo*. Such receptor-activating peptides yield information that is more readily interpreted than information obtained by administering proteolytic enzymes, which can cause many effects in addition to the activation of PAR$_2$. Unfortunately, potent and selective PAR$_2$ antagonists are not yet available either to verify the target (presumably PAR$_2$) of the PAR$_2$APs that we have used or to assess the precise contribution of PAR$_2$ itself in the inflammatory response of tissues to proteinases.

In summary, our data obtained with the PAR$_2$APs suggest that PAR$_2$ activation can cause a profound inflammatory response, characterized by oedema and granulocyte infiltration. The mechanism of the early inflammatory reaction induced by PAR$_2$ activation is largely independent of mast cell activation and of the production of prostaglandins and nitric oxide.

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