Modulation of Capsaicin-Evoked Visceral Pain and Referred Hyperalgesia by Protease-Activated Receptors 1 and 2

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Abstract. Protease-activated receptors (PARs) 1 and 2 are expressed in capsaicin-sensitive sensory neurons, being anti- and pro-nociceptive, respectively. Given the possible cross talk between PAR-2 and capsaicin receptors, we investigated if PAR-2 activation could facilitate capsaicin-evoked visceral pain and referred hyperalgesia in the mouse and also examined the effect of PAR-1 activation in this model. Intracolonic (i.col.) administration of capsaicin triggered visceral pain-related nociceptive behavior, followed by referred hyperalgesia. The capsaicin-evoked visceral nociception was suppressed by intraperitoneal (i.p.) TFLLR-NH₂, a PAR-1-activating peptide, but not FTLLR-NH₂, a control peptide, and unaffected by i.col. TFLLR-NH₂, SLIGRL-NH₂, a PAR-2-activating peptide, but not LRGILS-NH₂, a control peptide, administered i.col., facilitated the capsaicin-evoked visceral nociception 6 – 18 h after administration, while i.p. SLIGRL-NH₂ had no effect. The capsaicin-evoked referred hyperalgesia was augmented by i.col. SLIGRL-NH₂, but not LRGILS-NH₂, 6 – 18 h after administration, and unaffected by i.p. SLIGRL-NH₂ and i.p. or i.col. TFLLR-NH₂. Our data suggest that PAR-1 is antinociceptive in processing of visceral pain, whereas PAR-2 expressed in the colonic luminal surface, upon activation, produces delayed sensitization of capsaicin receptors, resulting in facilitation of visceral pain and referred hyperalgesia.

Keywords: protease-activated receptor, visceral pain, capsaicin, referred hyperalgesia

Introduction

Protease-activated receptors (PARs), a family of G protein-coupled seven-trans-membrane domain receptors, mediate cellular actions of certain serine proteases, consisting of four family members (PARs 1 to 4) (1 – 5). Activation of PARs occurs by proteolytic unmasking of the N-terminal extracellular tethered ligand that subsequently binds to the extracellular loop 2 of the receptor itself (6, 7). PAR-1, PAR-3, and PAR-4 are activated by thrombin, whereas PAR-2 is activated by trypsin, mast cell tryptase and coagulation factors VIIa and Xa, but not thrombin (6, 7). PAR-1, PAR-2, and PAR-4, but not PAR-3, are also non-enzymatically activated by synthetic peptides based on the N-terminal sequences of the tethered ligands; for example, SFLLR(-NH₂) and SLIGRL(-NH₂) for human PAR-1 and murine PAR-2, respectively (6, 7).

PAR-1 and PAR-2 play physiological/pathophysiological roles in a variety of tissues and cells, especially in the alimentary tract (8 – 12). PAR-1 and PAR-2 are also expressed in capsaicin-sensitive sensory neurons and, upon activation, capable of releasing neurotransmitters (13, 14). Intraplantar administration of PAR-2 agonists causes nociceptive behavior such as licking/biting and thermal, but not mechanical, hyperalgesia, accompanied by expression of spinal Fos protein, a marker for persistent activation of nociceptive neurons, in rats (15, 16). Vergnolle et al. (17) have independently reported that PAR-2 activation triggers both mechanical and thermal
hyperalgesia in rats or mice. The mechanisms of the nociception and/or hyperalgesia triggered by activation of peripheral PAR-2 involve activation of the glutamate-NMDA receptor pathway (18) and substance P-NK1 receptor pathway (17) in the spinal cord. Of special interest is that the PAR-2-triggered thermal hyperalgesia might occur through trans-activation or sensitization of capsaicin receptors by activation of PAR-2 on the C-fiber terminal (19), being consistent with the in vitro evidence for cross talk between capsaicin receptors and multiple G-protein-coupled receptors (20, 21). In contrast, intraplantar administration of PAR-1 agonists suppresses carrageenan-induced inflammatory hyperalgesia (22, 23).

PAR-2 is also involved in visceral pain/hyperalgesia. Hoogerwerf et al. (24) have demonstrated that the PAR-2-activating peptide SLIGRL-NH₂, administered into the pancreatic duct, increases the capsaicin-evoked spinal Fos expression in anesthetized rats, being in agreement with our previous evidence for trans-activation/sensitization of capsaicin receptors via PAR-2 in rat hindpaw (19). Intracolonic (i.col.) administration of SLIGRL-NH₂ at subinflammatory doses produces a delayed rectal mechanical hyperalgesia in conscious rats, as measured by counting abdominal contractions in response to rectal distension (25).

Most recently, Laird et al. (26) have developed a new model of visceral pain/hyperalgesia caused by i.col. capsaicin in unanesthetized mice. Given the accumulating information concerning the roles for PAR-2 and PAR-1 in pain modulation, including the possible cross talk between PAR-2 and capsaicin receptors (19, 24, 27), we thus investigated effects of i.col. and intraperitoneal (i.p.) administration of PARs-related peptides on the capsaicin-evoked visceral pain and referred hyperalgesia in mice. It is well known that i.p. administration of various chemicals including acetic acid produces writhing behavior, an abdominal pain-like response, implying that C-fiber stimulation with i.p. PAR agonists might produce or modulate nociception. Taken together with evidence that serosal and luminal application of PAR agonists in isolated intestinal preparations produces different effects on intestinal ionic transport (28), we applied PAR agonists through both i.p. and i.col. routes. Here, we provide evidence that PAR-2 activation in the lumen of mouse colon causes delayed facilitation of the capsaicin-evoked visceral nociception, whereas systemic PAR-1 activation is considered antinociceptive in this model.

Materials and Methods

Animals
Male ddY mice (20–25 g) were purchased from Japan SLC., Inc. (Hamamatsu), and used for experiments after 24-h fast. All studies were approved by the Kinki University School of Pharmaceutical Sciences’ Committee for the Care and Use of Laboratory Animals.

Chemicals
The following peptides were used: the PAR-1-activating peptide TFLLR-NH₂, which is much more specific for PAR-1 than the original human PAR-1-derived peptide SFLLR-NH₂ (6); the partially reversed PAR-1-inactive peptide FTLLR-NH₂; the PAR-2-activating peptide SLIGRL-NH₂; and the reversed PAR-2-inactive peptide LRGILS-NH₂. All peptides were synthesized by a solid-phase method, and their composition and purity were ascertained by HPLC analysis, amino acid analysis, and mass spectrometry. Capsaicin was purchased from Sigma (St. Louis, MO, USA), and amastatin was obtained from the Peptide Institute, Inc. (Minoh). PAR-related peptides and amastatin were dissolved in saline. Capsaicin was dissolved in a solution containing 10% ethanol, 10% Tween 80, and 80% saline.

Assessment of visceral nociception and referred hyperalgesia in mice

Intracolonic administration and basic protocol: The capsaicin-evoked visceral nociception test was performed as described previously (26) with minor modifications. Briefly, mice were placed on a raised wire mesh, under a clear plastic box (23.5 × 16.6 × 12.4 cm) and acclimated to the experimental environment for 30 min. Using a cannula with a rounded tip (external diameter: 1.6 mm), capsaicin at 164 and 491 nmol/mouse, in a volume of 50 μl (0.1% and 0.3% solution, respectively), was administered into the colon at 4 cm from the anus in the mouse, after application of vaseline in the perianal area to avoid the stimulation of somatic areas by contact with capsaicin. Immediately after administration of capsaicin, visceral pain-related nociceptive behavior was observed for 30 min, and mechanical hyperalgesia was assessed by use of von Frey filaments (see below). PAR-related peptides at 25 and 250 nmol/mouse were also administered i.col. to mice in the same manner, in order to evaluate their own effects on nociception. The intracolonic doses of SLIGRL-NH₂ were decided on the basis of the previous reports (29, 30), and the equivalent doses of TFLLR-NH₂ were employed, since these two peptides activate the target receptors in the same dose range.
even in vivo (11).

**Measurement of visceral pain-related nociceptive behavior:** Visceral pain-related nociceptive behavior was defined as licking of the abdomen, stretching, squashing of the lower abdomen against the floor, and abdominal retraction (26). The number of these behavioral responses were measured every 5 min for 30 min immediately after i.col. administration of stimulants. The data are shown as the number of responses every 5 min or for 30 min.

**Determination of referred hyperalgesia:** Three distinct von Frey filaments with strengths of 0.02, 0.16, and 1.0 g were used to stimulate the lower to mid abdomen of the mouse. The mechanical stimulation with 3 distinct filaments, in an ascending order of the strength, was applied at intervals of 5 – 10 s, 10 times for each filament, 30 – 40 min after i.col. administration of capsaicin. Two successive stimuli to the same point were avoided, considering ‘wind-up’ effects or desensitization. Scoring of nociceptive behavior was defined as follows: score 2 = strong retraction of the abdomen or jumping; score 1 = licking/scratching of the abdomen, immediate escape or licking or scratching of the site stimulated with filaments; score 0 = no response. The data are expressed as the mean score of the responses caused by challenge with each filament.

**Schedule of administration of PAR-related peptides:** PAR-related peptides were administered i.p. or i.col. alone or 5 min after i.p. amastatin, an inhibitor of aminopeptidase that degrades peptides, at 2.5 μmol/kg. Capsaicin was administered i.col. immediately or 1, 6, or 18 h after i.p. or i.col. administration of the peptides.

**Statistical analyses**

Data are shown as the mean with S.E.M. Statistical significance was evaluated by ANOVA followed by Tukey’s multiple comparison test or Student’s t-test for two-group data, and it was set at a P<0.05 level.

**Results**

**Visceral nociceptive behavior caused by i.col. administration of capsaicin, but not the PAR-1-activating peptide TFLLR-NH₂ or the PAR-2-activating peptide SLIGRL-NH₂ in mice**

Although i.col. administration of the vehicle elicited some responses, i.col. capsaicin at 491 nmol/mouse induced significant increase in visceral nociceptive behavior, at least for 30 min (Fig. 1A). The total nociceptive behavior for 30 min was significantly augmented by capsaicin at 164 and 491 nmol/mouse (Fig. 1B). A larger dose, 820 nmol/mouse, of capsaicin evoked uncountable severe responses such as convulsion of the whole body (data not shown). On the other hand, neither the PAR-1-activating peptide TFLLR-NH₂ nor the PAR-2-activating peptide SLIGRL-NH₂, administered at i.col. 25 and 250 nmol/mouse alone or in combination with i.p. amastatin at 2.5 μmol/kg, elicited nociceptive behavior (Fig. 2).

**Suppression of the capsaicin-evoked visceral pain-related nociceptive behavior by i.p., but not i.col., administration of the PAR-1-activating peptide TFLLR-NH₂**

Intracolonic administration of the PAR-1 agonist
TFLLR-NH₂ at 25 and 250 nmol/mouse in combination with i.p. amastatin had no effect on the nociceptive behavior caused by a high dose, 491 nmol/mouse, of capsaicin (Fig. 3A). On the other hand, i.p. administration of TFLLR-NH₂ at 5 μmol/kg in combination with amastatin suppressed the capsaicin-induced nociceptive behavior, although it had no effect without amastatin (Fig. 3B). The PAR-1-inactive control peptide FTLLR-NH₂ (Fig. 3B) or the PAR-2 agonist SLIGRL-NH₂ (Fig. 3C), administered i.p. at 5 μmol/kg in combination with amastatin, failed to alter the capsaicin-evoked nociception.

**Delayed facilitation of the capsaicin-evoked visceral pain-related nociceptive behavior by i.col., but not i.p., administration of the PAR-2-activating peptide SLIGRL-NH₂**

Next, we examined whether receptor-activating peptides for PAR-2 could facilitate the visceral nociception caused by capsaicin. Intracolonic administration of SLIGRL-NH₂ at 25 nmol/mouse in combination with i.p. amastatin significantly augmented the nociceptive behavior induced by i.col. capsaicin at a low dose, 164 nmol/mouse, 6 and 18 h, but not 1 h, after administration, although it did not modify non-specific responses caused by vehicle for capsaicin at the corresponding time points (Fig. 4A). In contrast, SLIGRL-NH₂, administered in the same manner, did not alter after the nociceptive behavior induced by i.col. capsaicin at a high dose, 491 nmol/mouse (Fig. 4B). Neither i.col. SLIGRL-NH₂ at the low dose of 8.3 nmol/mouse nor the PAR-2-inactive control peptide LRGILS-NH₂ at 25 nmol/mouse significantly altered the nociceptive behavior induced by the low dose of capsaicin 6 and 18 h after administration (Fig. 5: A and B). Intraperitoneal administration of SLIGRL-NH₂ at 5 μmol/kg or i.col. administration of TFLLR-NH₂ at 25 nmol/mouse had no effect on the capsaicin-induced nociceptive behavior 18 h after administration (Fig. 5C). Collectively, i.col. SLIGRL-NH₂ caused delayed facilitation of capsaicin-evoked nociception, while neither i.p. SLIGRL-NH₂ nor i.col. TFLLR-NH₂ revealed significant effect.

**Effects of i.col. capsaicin, i.p. TFLLR-NH₂, or i.col. SLIGRL-NH₂ on nociceptive responses to stimuli with von Frey filaments**

Intracolonic capsaicin at 491 nmol/mouse, but not 164 nmol/mouse, induced referred hyperalgesia in mice 30 min after administration when challenged with filaments of 0.16- or 1.00-g strength (Fig. 6A). We next examined effects of i.col. TFLLR-NH₂ and i.col. SLIGRL-NH₂ themselves in the von Frey test. Intraperitoneal administration of TFLLR-NH₂ at 5 μmol/kg in combination with amastatin that suppressed capsaicin-evoked visceral nociception (Fig. 3B) failed to alter nociceptive responses to stimulation with von Frey filaments, immediately or 30 min after administration (Fig. 6B). Intracolonic administration of SLIGRL-NH₂ at 25 nmol/mouse in combination with amastatin that facilitated capsaicin-evoked visceral nociception (Figs. 4A, 5B) did not alter mechanical nociception in response to von Frey filaments (Fig. 6C).
Facilitation by i.col., but not i.p., administration of SLIGRL-NH$_2$ of the capsaicin-evoked referred hyperalgesia

Intracolonic administration of SLIGRL-NH$_2$ at 25 nmol/mouse, but not 8.3 nmol/mouse, in combination with i.p. amastatin facilitated the capsaicin-evoked referred hyperalgesia at 6 or 18 h (Fig. 7A). In contrast, the reversed peptide LRGILS-NH$_2$, given i.col. at 25 nmol/mouse, produced no significant effect at 6 h (Fig. 7B). Surprisingly, intraperitoneal administration of TFLLR-NH$_2$ at 5 μmol/kg failed to alter the capsaicin-evoked referred hyperalgesia (Fig. 7C), being inconsistent with the finding that it suppressed the capsaicin-evoked spontaneous nociceptive behavior (Fig. 3B).

Discussion

In the present study, i.p. administration of the PAR-1-activating peptide TFLLR-NH$_2$ suppressed the capsaicin-evoked visceral pain, whereas i.col. administration of the PAR-2-activating peptide SLIGRL-NH$_2$ caused delayed facilitation of the capsaicin-evoked visceral pain and referred hyperalgesia, as summarized in Table 1.

PAR-2 is expressed in capsaicin-sensitive sensory neurons and, upon activation, releases several neurotransmitters such as calcitonin gene-related peptide (CGRP) and substance P, resulting in neurogenic inflammation and pain/hyperalgesia in rat hindpaw and gastric mucus secretion accompanied by mucosal cytoprotection in rat stomach (10, 13, 15, 17). Recently, we have found that the PAR-2-triggered thermal hyperalgesia in the hindpaw and mucus secretion in the stomach are inhibited by capsazepine, a capsaicin-receptor antagonist, suggesting involvement of trans-activation of capsaicin receptors by activation of PAR-2, a G-protein-coupled receptor, in the C-fiber terminal (15, 19, 27), as described by Premkumar and Ahern (20) and by Tominaga et al. (21). Therefore, it is likely that, in the present study, the PAR-2 agonist SLIGRL-NH$_2$, given i.col., might directly sensitize capsaicin receptors expressed on the luminal surface in the colon, facilitating capsaicin-evoked visceral pain and concomitant referred hyperalgesia in mice. This notion is consistent with the previous evidence that subinflammatory doses of SLIGRL-NH$_2$, given i.col., produced a delayed rectal hyperalgesia in rats, as evaluated by monitoring abdominal contraction in response to rectal distention (25). However, considering the delayed onset in the present and previous (25) studies, the PAR-2-mediated visceral nociceptive modulation might involve some inflammatory mechanisms. Actually, i.col. administration of SLIGRL-NH$_2$ at doses equivalent to those employed in the present study causes colonic inflammatory reactions in mice characterized by granulocyte infiltration, increased wall thickness, tissue damage, and elevated T-helper cell type 1 cytokine, which develop.
Fig. 4. Time-related facilitation of the capsaicin-evoked visceral nociception by intracolonic (i.col.) administration of SLIGRL-NH₂ in mice. SLIGRL-NH₂ (SLp-NH₂) at 25 nmol/mouse or vehicle in combination with i.p. amastatin at 2.5 μmol/kg was administered i.col. 1, 6, 18 h before i.col. capsaicin at 164 (A) or 491 (B) nmol /mouse. Data show the mean with S.E.M. from 4 – 12 mice. *P<0.05, **P<0.01 vs vehicle + capsaicin.

Fig. 5. Delayed effects of PAR-related peptides on the capsaicin-evoked visceral nociception in mice. SLIGRL-NH₂ (SLp-NH₂), LRGILS-NH₂ (LRp-NH₂), or vehicle (V) in combination with i.p. amastatin (Ama) at 2.5 μmol /kg was administered i.col. 6 h (A) or 18 h (B) before i.col. capsaicin. C: SLIGRL-NH₂ and TFLLR-NH₂ (TFp-NH₂) in combination with i.p. amastatin were administered i.p. and i.col., respectively, 18 h before i.col. capsaicin. Data show the mean with S.E.M. from 6 – 12 (A), 20 – 26 (B, left), 9 (B, right), or 6 (C) mice. *P<0.05 vs vehicle.
and persist 4–24 h after i.col. administration (29). Both capsaicin-sensitive sensory neurons and nitric oxide (NO) appear to be involved in the PAR-2-mediated colonic inflammation (30). Given that various inflammatory and/or nociceptive mediators including bradykinin and ATP cause sensitization or trans-activation of capsaicin receptors in the sensory neurons (20, 21), it is likely that PAR-2 activation might induce release of these mediators, which would activate their own receptors in capsaicin-sensitive neurons and thereby facilitate

![Diagram of nociceptive responses to mechanical stimulation with von Frey filaments in mice.](image)

**Fig. 6.** Effects of intracolonic (i.col.) capsaicin, i.p. TFLLR-NH$_2$ or i.col. SLIGRL-NH$_2$ on nociceptive responses to mechanical stimulation with von Frey filaments in mice. A: Capsaicin was administered i.col. 30 min before the von Frey test. B: TFLLR-NH$_2$ (TFp-NH$_2$) and amastatin at 2.5 μmol/kg was co-administered i.p. immediately or 30 min before the von Frey test. C: SLIGRL-NH$_2$ (SLp-NH$_2$) in combination with i.p. amastatin at 2.5 μmol/kg was administered i.col. 18 h before the von Frey test. Data show the mean with S.E.M. from 6–9 mice. **P<0.01 vs vehicle.

![Diagram of nociceptive responses to mechanical stimulation with von Frey filaments in mice.](image)

**Fig. 7.** Delayed effects of PAR-related peptides on the capsaicin-evoked referred hyperalgesia in mice. SLIGRL-NH$_2$ (SLp-NH$_2$) (A) or LRGILS-NH$_2$ (LRp-NH$_2$) (B) in combination with i.p. amastatin at 2.5 μmol/kg was administered i.col. 6 h or 18 h before i.col. capsaicin at 164 nmol/mouse (Cap, 164). C: TFLLR-NH$_2$ (TFp-NH$_2$) and amastatin were co-administered i.p. immediately before i.col. capsaicin at 491 nmol/mouse (Cap, 491). The von Frey test was performed 30 min after i.col. capsaicin. Data show the mean with S.E.M. from 6 (vehicle + Cap, 164) or 8–15 (SLIGRL-NH$_2$ + Cap, 164) mice in (A, left) and from 6 (A, right), 5 (B), or 18 (C) mice. *P<0.05, **P<0.01 vs vehicle.
capsaicin-evoked visceral nociception and referred hyperalgesia.

Intraplantar administration of SLIGRL-NH₂ triggers spontaneous nociceptive behavior such as licking and biting, in addition to thermal hyperalgesia, in rats and mice (15, 18). Intracolonic administration of SLIGRL-NH₂ is also capable of producing a delayed rectal hyperalgesia to distension stimuli in rats (25). Nonetheless, i.col. administration of SLIGRL-NH₂ itself failed to trigger visceral pain-related nociceptive behavior or referred hyperalgesia in mice, although it facilitated the nociceptive effects of i.col. capsaicin, in the present study. Similarly, Hoogerwerf et al. (24) have described that SLIGRL-NH₂ itself is incapable of producing release of CGRP in cultured dorsal root ganglion neurons in vitro in rats, while it facilitated those effects caused by capsaicin. Thus, direct and/or indirect capsaicin receptor sensitization is considered a critical step for PAR-2 modulation of nociceptive processing, although involvement of other pathways cannot be ruled out.

Considering that a supramaximal dose, 820 nmol/mouse, of capsaicin evoked uncountable severe responses such as convulsion of the whole body, it might be questioned why combination of intracolonic PAR-2 agonist and capsaicin at 491 nmol/mouse did not induce such behavior. It is likely that PAR-2 activation would induce sensitization to capsaicin, possibly due to receptor sensitization, but not increase in maximal responses to capsaicin. This is in agreement with the in vitro evidence that capsaicin receptors can be sensitized by a cross talk with some G protein-coupled receptors, which does not alter the maximal responses to capsaicin (20, 21).

The finding that i.p., but not i.col., administration of the PAR-1 agonist TFLLR-NH₂ suppressed capsaicin-evoked visceral nociceptive behavior is in agreement with the analgesic effect of intraplantar TFLLR-NH₂ on carrageenin-induced hyperalgesia in rat hindpaw (22, 23). The exact reason why i.p., but not i.col., TFLLR-NH₂ attenuated capsaicin-evoked visceral nociceptive behavior has yet to be elucidated. It can be speculated that TFLLR-NH₂ given i.p. could be distributed systemically, while TFLLR-NH₂ given i.col. would reach the luminal surface and surrounding regions in the colon, but not distant tissues. Our data, in turn, might suggest that activation of PAR-1 in tissues other than colonic mucosa suppressed i.col. capsaicin-evoked nociception. TFLLR-NH₂, administered systemically, is not considered to reach the central nervous system (CNS), although activation of PAR-1 in the CNS also exhibits antinociceptive activity (31). Peripheral PAR-1 would thus appear to play an antinociceptive role in processing of not only somatic but also visceral pain. It was surprising that TFLLR-NH₂ had no effect on the capsaicin-evoked referred hyperalgesia. Hence, mechanisms for the visceral nociception and referred hyperalgesia caused by capsaicin might be distinct, although the reasons are still open to question.

In summary, activation of PAR-2 expressed by the colonic luminal surface, upon activation, produces delayed sensitization of capsaicin receptors, resulting in facilitation of visceral pain and referred mechanical hyperalgesia. In contrast, peripheral PAR-1 activation appears to play an antinociceptive role in processing of visceral pain.

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References