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Suppression of pancreatitis-related allodynia/hyperalgesia by proteinase-activated receptor-2 in mice

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1 Proteinase-activated receptor-2 (PAR2), a receptor activated by trypsin and tryptase, is abundantly expressed in the gastrointestinal tract including the C-fiber terminal, and might play a role in processing of visceral pain. In the present study, we examined and characterized the roles of PAR2 in pancreatitis-related abdominal hyperalgesia/allodynia in mice.

2 Caerulein, administered i.p. once, caused a small increase in abdominal sensitivity to stimulation with von Frey hairs, without causing pancreatitis, in PAR2-knockout (KO) mice, but not wild-type (WT) mice.

3 Caerulein, given hourly six times in total, caused more profound abdominal hyperalgesia/allodynia in PAR2-KO mice, as compared with WT mice, although no significant differences were detected in the severity of pancreatitis between the KO and WT animals.

4 The PAR2-activating peptide, 2-furoyl-LIGRL-NH_2, coadministered repeatedly with caerulein six times in total, abolished the caerulein-evoked abdominal hyperalgesia/allodynia in WT, but not PAR2-KO, mice. Repeated doses of 2-furoyl-LIGRL-NH_2 moderately attenuated the severity of caerulein-induced pancreatitis in WT animals.

5 Our data from experiments using PAR2-KO mice provide evidence that PAR2 functions to attenuate pancreatitis-related abdominal hyperalgesia/allodynia without affecting pancreatitis itself, although the PAR2AP applied exogenously is not only antinociceptive but also anti-inflammatory.
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Abbreviations: 2-furoyl-LIGRL-NH_2, 2-furoyl-Leu-Ile-Gly-Arg-Leu-amide; IL, interleukin; KO, knockout; L-NAME, N^G-nitro-L-arginine methyl ester; PAR2, proteinase-activated receptor-2; PAR2AP, PAR2-activating peptide; SLIGRL-NH_2, Ser-Leu-Ile-Gly-Arg-Leu-amide; TRPV-1, transient receptor potential vanilloid-1; WT, wild-type

Introduction

Proteinase-activated receptor-2 (PAR2), a receptor for trypsin, is abundantly distributed throughout the alimentary systems, modulating various functions (Saifeddine *et al*., 1996; Cocks *et al*., 1999; Nguyen *et al*., 1999; Kawabata *et al*., 2000; 2001b, c; Cuffe *et al*., 2002; Kawao *et al*., 2002a; Nishikawa *et al*., 2002). PAR2 is also present in the C-fiber neurons, participating in neurogenic inflammation (Steinhoff *et al*., 2000) and somatic pain/hyperalgesia (Kawabata *et al*., 2001a; 2002a; Vergnolle *et al*., 2001). The PAR2-triggered somatic hyperalgesia might involve sensitization of transient receptor potential vanilloid-1 (TRPV-1) receptors (capsaicin receptors) present in the C-fiber terminal (Kawao *et al*., 2002b), through activation of protein kinase C (Amadesi *et al*., 2004; Dai *et al*., 2004). In the gastrointestinal tract, PAR2 in C-fibers appears to exert mucosal cytoprotection (Fiorucci *et al*., 2001; Kawabata *et al*., 2001b; 2005). Nonetheless, intracolonic (i.col.) administration of PAR2 agonists appears to cause colitis in wild-type (WT), but not PAR2-knockout (KO), mice (Cenac *et al*., 2002). On the other hand, i.col. subinflammatory doses of PAR2 agonists cause delayed development of hypersensitivity to rectal distention in rats (Coelho *et al*., 2002). Similarly, i.col. administration of the PAR2-activating peptide (PAR2AP), SLIGRL-NH_2, produces delayed hypersensitivity to i.col. capsaicin in ddY mice (Kawao *et al*., 2004). Apart from the stimulative roles for PAR2 in pancreatic exocrine secretion (Nguyen *et al*., 1999; Kawabata *et al*., 2000), two independent studies have revealed that systemic administration of PAR2 agonists protects against caerulein-induced pancreatitis in rats and mice (Namkung *et al*., 2004; Sharma *et al*., 2005) and that PAR2-KO mice exhibit more profound pancreatitis than WT animals (Sharma *et al*., 2005), implying a protective role for PAR2 during pancreatitis. Nevertheless, infusion of subinflammatory doses of PAR2 agonists into the pancreatic duct produces behavioral pain responses accompanied by expression of Fos, a marker of neuronal activation, in the thoracic spinal dorsal horn in rats (Hoogerwerf *et al*., 2001; 2004). Thus, PAR2 could be anti-inflammatory but pronociceptive in the pancreas.

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To clarify the role of PAR2 in pancreatic nociceptive processing during pancreatitis, the present study investigated effect of genetic PAR2 deficiency and the PAR2AP administered systemically on abdominal allodynia/hyperalgesia related to caerulein-evoked pancreatitis in mice.

Methods

Animals

Male ddY mice weighing 20–25 g were purchased from Japan SLC Inc. (Shizuoka, Japan). Female WT ($PAR2^{+/+}$) and PAR2-KO ($PAR2^{-/-}$) mice of C57BL/6 background were provided from Kowa Company (Tokyo, Japan). The PAR2-KO strain was prepared as described previously (Ferrell *et al*., 2003), and maintained by backcrossing heterozygous ($PAR2^{+/-}$) males with C57BL/6 females at each generation. The genotype of the mice was confirmed by Southern blot analysis and PCR analysis of DNA obtained from tail biopsy. Homozygous ($PAR2^{-/-}$) and WT ($PAR2^{+/+}$) female mice generated from male and female $PAR2^{+/-}$ mice at backcross generation 8 were used at 8–12 weeks of age for the experiments. All animals were used with approval by the Kinki University School of Pharmaceutical Sciences' Committee for the Care and Use of Laboratory Animals, on the basis of Guiding Principles for the Care and Use of Laboratory Animals Approved by The Japanese Pharmacological Society. In particular, every effort was made to minimize the number of animals used per experiment and the number of experiments performed.

Evaluation of caerulein-evoked pancreatitis and related mechanical abdominal allodynia/hyperalgesia in mice

Caerulein at 50 $\mu g\,kg^{-1}$ was administered i.p. to mice once or six times at 1-h intervals. At 30 min after a single dose or the final dose of caerulein or vehicle, abdominal sensitivity of each mouse to mechanical stimuli was determined using von Frey filaments with strengths of 0.02, 0.16, and 1.0 g. The mouse was placed on a raised wire mesh floor under a clear plastic box ($23.5 \times 16.6 \times 12.4$ cm), as mentioned above, and the upper abdomen was stimulated with three distinct filaments in an ascending order of the strength. The mechanical stimulation with each filament was applied five times at intervals of 5–10 s, and, after a 1-min resting period, another five times in the same manner, namely 10 times in total. Successive twice stimuli to the same point were avoided, considering 'wind-up' effects or desensitization. Scoring of nociceptive behavior was defined as follows: score 0 = no response; score 1 = immediate escape or licking/scratching of the site stimulated with von Frey hairs; score 2 = strong retraction of the abdomen or jumping. The data are expressed as the total score of responses caused by 10-time challenges with each hair.

For evaluation of the severity of caerulein-evoked pancreatitis, blood samples were collected from the abdominal aorta in the mice treated with caerulein or vehicle under urethane (1.5 $g\,kg^{-1}$, i.p.) anesthesia, and the pancreata were excised and weighed. It is of note that i.p. administration of urethane itself did not induce any pathological changes in the pancreatic tissues in our experimental conditions. Plasma amylase activity was determined by the colorimetric method using an assay kit (Amylase-Test Wako, Wako Pure Chem., Osaka, Japan). The pancreata were fixed, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The pancreas sections were examined and scored morphologically by an experienced pancreatic morphologist, who was not aware of the sample identity. Morphological scoring was performed in terms of acinar cell death, interstitial edema and neutrophil infiltration. The criteria for scoring of acinar cell death was as follows: score 0 = 0–1 necrosis of acinar cells per microscopic field ($\times 400$); score 1 = 2–5 necrotic cells; score 2 = more than 5 necrotic cells; score 3 = acinar cell death accompanied by the destruction of the histoarchitecture of whole or parts of the acini. The extent of interstitial edema and neutrophil infiltration was scored as follows: score 0 = none; score 1 = mild; score 2 = moderate; score 3 = severe.

Drug administration

A potent, aminopeptidase-resistant and highly specific PAR2AP, 2-furoyl-LIGRL-NH_2 (Kawabata *et al*., 2004), at 0.1 $\mu mol\,kg^{-1}$ was coadministered i.p. hourly with caerulein six times in total to WT or PAR2-KO mice. We have shown that this dose of 2-furoyl-LIGRL-NH_2, given i.p., was maximally effective in the gastric mucosal protection study (Kawabata *et al*., 2005) and the salivation assay (Kawabata *et al*., 2004) in mice.

In inhibition experiments, N^G-nitro-L-arginine methyl ester (L-NAME) at 30 $mg\,kg^{-1}$ was administered i.p. to mice 30 min before the first dose of caerulein. For ablation of capsaicin-sensitive sensory nerves, the mice received subcutaneous (s.c.) administration of capsaicin in doses of 25, 50, and 50 $mg\,kg^{-1}$ (125 $mg\,kg^{-1}$ in total), three times, at 0, 6, and 32 h, respectively. Before each dose of capsaicin, the mouse was anesthetized with i.p. pentobarbital at 45 $mg\,kg^{-1}$. The mice were used for experiments 10–13 days after the last dose of capsaicin. In the preliminary experiments, the efficacy of capsaicin treatment was verified by monitoring the wiping reflex to ocular instillation of 10 μl of 0.1 mM capsaicin.

Chemicals

The PAR2AP, 2-furoyl-Leu-Ile-Gly-Arg-Leu-amide (2-furoyl-LIGRL-NH_2) was synthesized essentially according to a solid phase method and purified by high-performance liquid chromatography (HPLC), and the composition and purity were determined by mass spectrometry. Porcine trypsin, L-NAME and capsaicin were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.), and caerulein was from Bachem (Bubendorf, Switzerland). Capsaicin was dissolved in a solution containing 10% ethanol, 10% Tween 80, and 80% saline, and all other chemicals were dissolved in saline.

Statistics

Data are expressed as means with s.e.m., and analyzed statistically by Students' *t*-test for comparisons between two groups and by Tukey's test for multiple comparisons. A probability value of <0.05 was regarded as significant.

Results

Caerulein-evoked pancreatitis and related mechanical abdominal allodynia/hyperalgesia in ddY mice and in WT and PAR2-KO mice of C57BL/6 background

Caerulein, administered repeatedly six times, caused significant or nonsignificant increases in nociceptive scores in response to mechanical stimuli of distinct strengths in ddY mice (Figure 1). Repeated doses of caerulein also produced significant enhancement of the score in response to 0.02 g stimulation, but not 0.16 or 1.0 g stimulation, in WT C57BL/6 mice (Figure 2b), indicating that C57BL/6 mice might be less sensitive to caerulein than ddY mice in this assay. Nonetheless, more profound increases in nociceptive scores in response to stimulation of three distinct strengths were induced by repeated administration of caerulein in PAR2-KO mice (Figure 2b). Repeated doses of caerulein caused typical pancreatitis as evaluated by increased plasma amylase activity and pancreatic weight and by morphological scoring (Figure 3). In spite of the increased abdominal hypersensitivity to mechanical stimuli in PAR2-KO mice (Figure 2b), the severity of repeated caerulein-evoked pancreatitis was not clearly different between WT and PAR2-KO mice of C57BL/6 background (Figure 3).

Surprisingly, even a single dose of caerulein significantly augmented nociceptive scores in response to stimulation with 0.02 and 0.16 g filaments in PAR2-KO mice, while it failed to alter abdominal sensitivity to mechanical stimuli in WT C57BL/6 animals (Figure 2a). Of importance is that a single dose of caerulein produced almost no symptoms of pancreatitis in either PAR2-KO or WT mice, although slight increase in plasma amylase activity was detected in PAR2-KO mice (Figure 3).

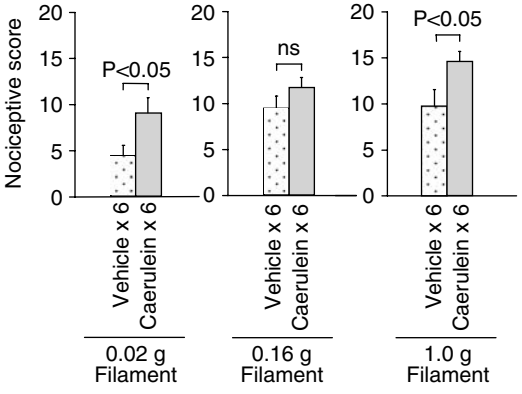

Figure 1 Caerulein-evoked abdominal mechanical allodynia/hyperalgesia in ddY mice. Caerulein was administered i.p. hourly six times in total to ddY mice. $n = 11$. ns, not significant.

Effect of systemic administration of the PAR2 agonist on caerulein-evoked pancreatitis and related mechanical abdominal allodynia/hyperalgesia in mice

The potent PAR2 agonist 2-furoyl-LIGRL-NH_2 at 0.1 μmol kg^{-1}, coadministered hourly with caerulein six times in total, completely blocked the caerulein-evoked mechanical abdominal allodynia/hyperalgesia in response to 0.02 g stimulation in WT C57BL/6 mice (Figure 4), and also in ddY mice (data not shown). In contrast, repeated coadministration of 2-furoyl-LIGRL-NH_2 at 0.1 μmol kg^{-1} with caerulein failed to inhibit the caerulein-evoked abdominal hypersensitivity in PAR2-KO mice (Figure 4).

Interestingly, 2-furoyl-LIGRL-NH_2, given repeatedly abolished the caerulein-evoked enhancement of plasma amylase activity in WT mice (Figure 5a), although it produced little effect on increased pancreatic weight (Figure 5b). Morphological examination also revealed mild protective effect of 2-furoyl-LIGRL-NH_2 against caerulein-evoked pancreatitis in WT animals (Figure 5c and d). Similar protection exerted by 2-furoyl-LIGRL-NH_2 against caerulein-evoked pancreatitis was also detected in ddY mice (data not shown). Finally, in inhibition experiments, neither pretreatment with the NO synthase inhibitor L-NAME at 30 mg kg^{-1} nor ablation of capsaicin-sensitive sensory neurons affected the inhibitory effect of 2-furoyl-LIGRL-NH_2 at 0.1 μmol kg^{-1} on the increased plasma amylase activity in the mice (data not shown).

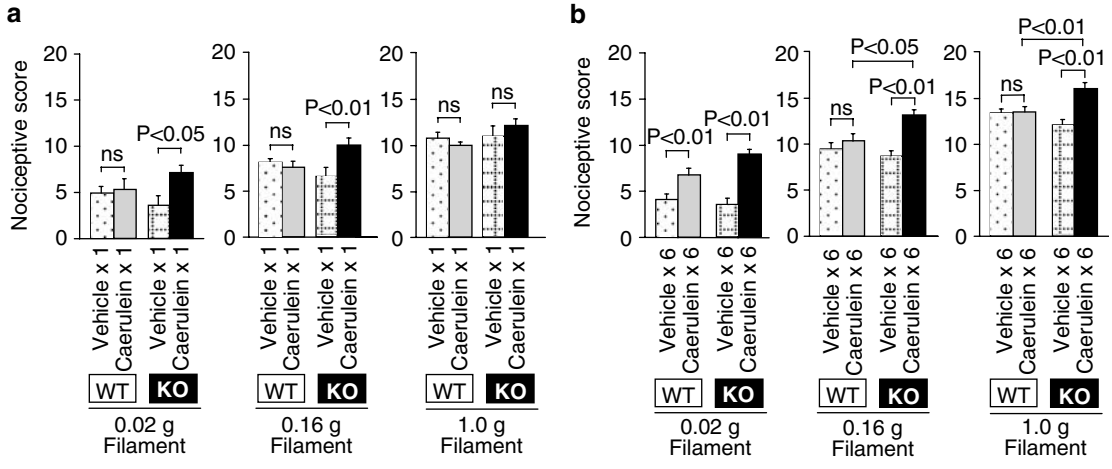

Figure 2 Caerulein-evoked abdominal mechanical allodynia/hyperalgesia in WT and PAR2-KO mice of C57BL/6 background. Caerulein was administered i.p. once (a) or hourly six times in total (b) to WT and PAR2-KO mice of C57BL/6 background. $n = 6$ (b) or 14–20 (a). ns, not significant. KO, PAR2-KO.

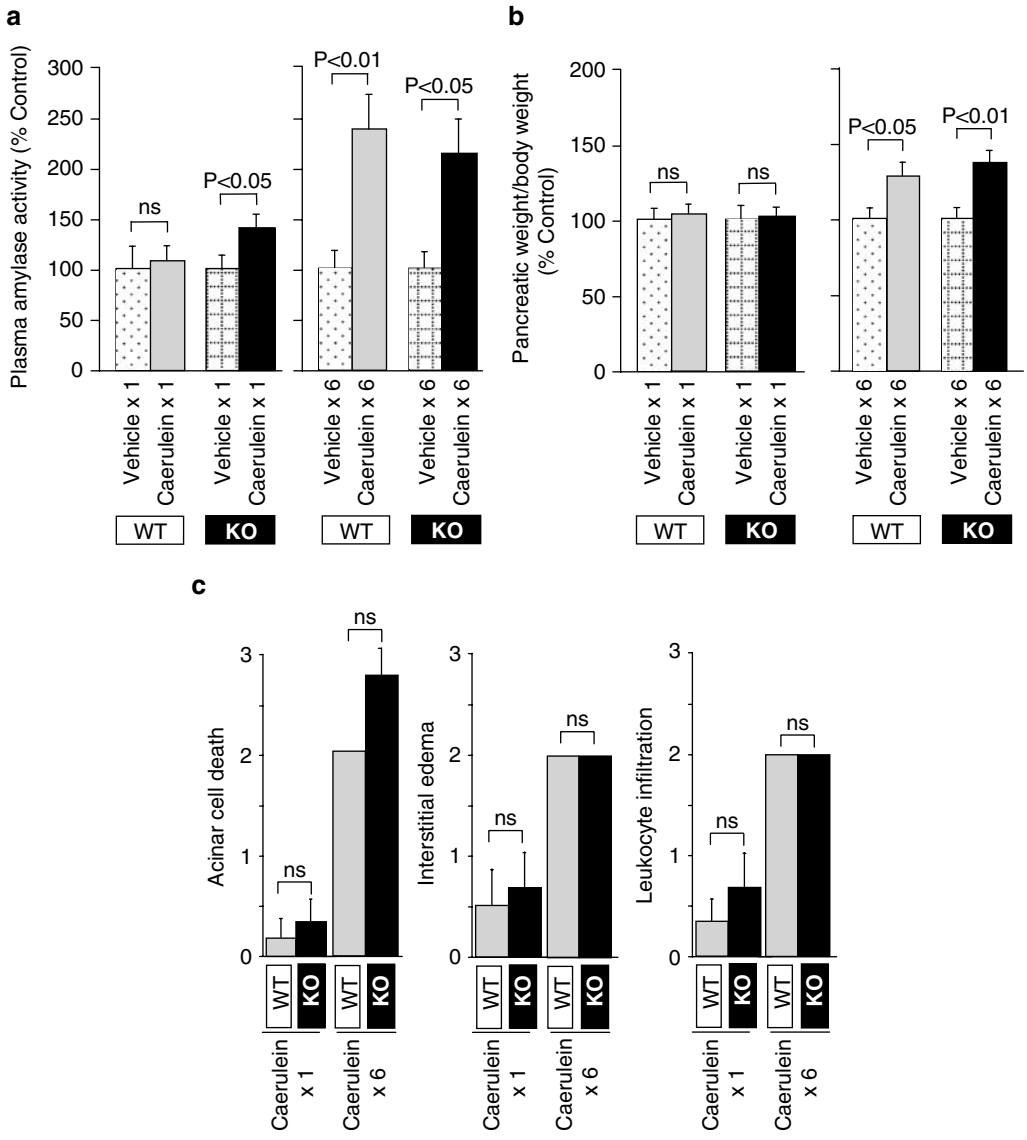

Figure 3 Severity of caerulein-evoked pancreatitis in WT and PAR2-KO mice of C57BL/6 background. Caerulein was administered i.p. hourly six times in total or once to WT and PAR2-KO mice of C57BL/6 background. The severity of pancreatitis was evaluated by increased plasma amylase activity (a), increased pancreatic wet weight (b) and morphological scoring of pancreatic sections (c). $n = 4$–6. ns, not significant. KO, PAR2-KO.

Discussion

In the present study, we found that a single dose or repeated doses of caerulein produced more profound abdominal mechanical allodynia/hyperalgesia in PAR2-KO mice than that in WT mice, although the severity of pancreatitis itself was not clearly different between WT and PAR2-KO animals. Our data thus imply potential antinociceptive roles of PAR2 during pancreatitis. This notion could be supported by the finding that the PAR2AP given exogenously abolished caerulein-evoked abdominal allodynia/hyperalgesia in WT, but not PAR2-KO, mice. Nonetheless, the anti-inflammatory property was also suggested by the finding that the PAR2AP also moderately suppressed the severity of caerulein-evoked pancreatitis.

It has been described that visceral pain can be elicited from the pancreas when the pancreas is inflamed or during pancreatic cancer. In humans, pain elicited from the pancreas is often referred to the upper abdominal area and radiates to the back, and these areas of the skin are usually tender to touch (referred mechanical allodynia) (Z'graggen *et al*., 1998). In animal models for pancreatitis, visceral pain is characterized as a lowered threshold of responses to mechanical stimulation of the abdominal areas (Vera-Portocarrero *et al*., 2003). Using these paradigms, the present study demonstrates the potential antinociceptive roles for PAR2 during pancreatitis in mice. This is in contrast with the nociceptive effect of PAR2 agonists injected into the pancreatic duct in the absence of pancreatitis in rats (Hoogerwerf *et al*., 2001; 2004), although the possibility can not be ruled out that species differences are simply responsible. On the other hand, PAR2 could also be anti-inflammatory in the pancreas, considering the protective effect of exogenously applied PAR2APs against experimental pancreatitis as described in the present and previous studies

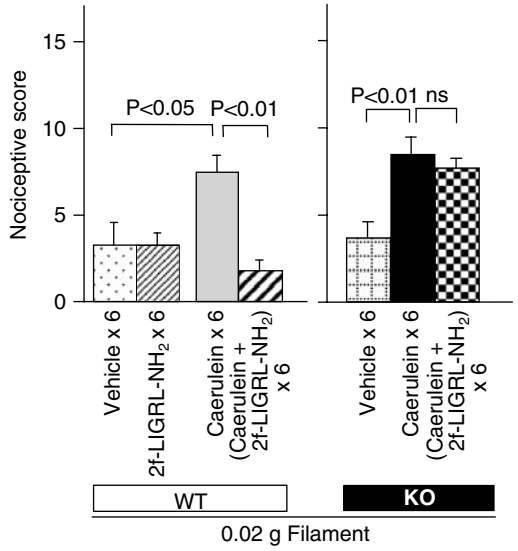

Figure 4 Effect of repeated administration of the PAR2AP on caerulein-evoked abdominal mechanical allodynia/hyperalgesia in WT or PAR2-KO mice of C57BL/6 background. The PAR2AP, 2-furoyl-LIGRL-NH_2 (2f-LIGRL-NH_2), at 0.1 μmol kg^{-1} was coadministered i.p. hourly with caerulein six times in total to WT or PAR2-KO mice. KO, PAR2-KO. $n = 5$–7. ns, not significant.

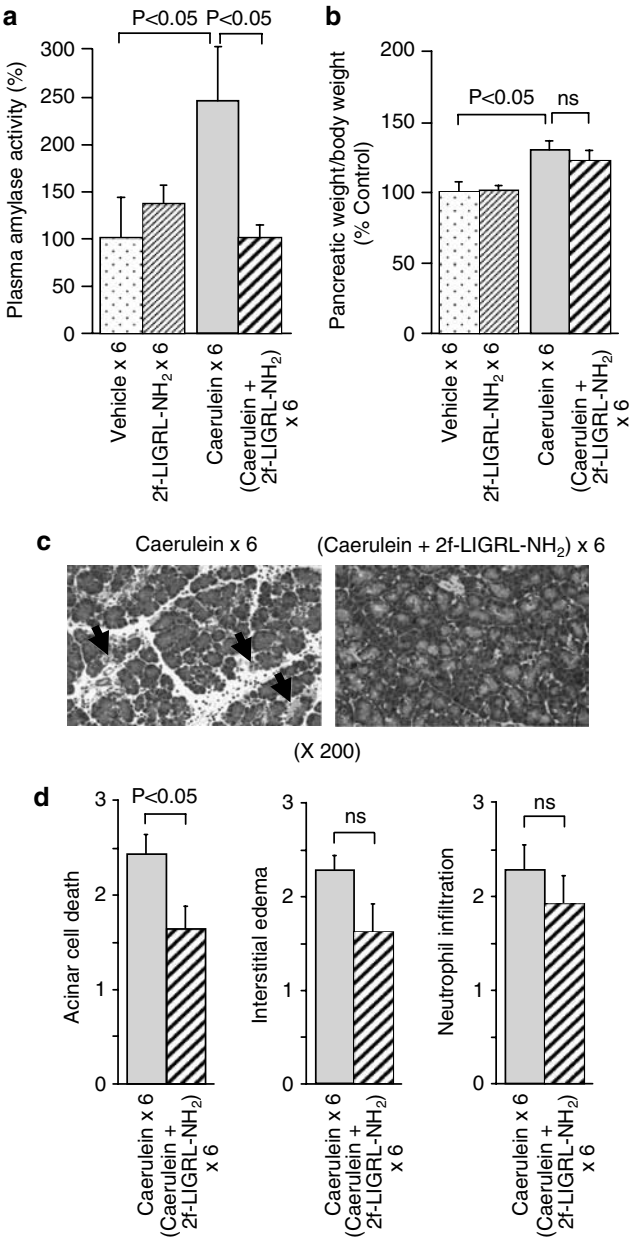

Figure 5 Effect of repeated administration of the PAR2AP on caerulein-evoked pancreatitis in WT mice of C57BL/6 background. (a) and (b) Effects of 2f-LIGRL-NH_2 on increased plasma amylase activity (a) and pancreatic wet weight (b) in the animals. $n = 6$–7. (c) Typical microphotographs of pancreatic tissues in the mice that received 6 hourly administration of caerulein alone and in combination with 2f-LIGRL-NH_2. Arrows indicate acinar cell death. (d) Morphological scoring of pancreatic sections from the mice treated with caerulein alone or in combination with 2f-LIGRL-NH_2. $n = 13$–14. ns, not significant.

(Namkung *et al*., 2004; Sharma *et al*., 2005). Nonetheless, no significant differences between WT and PAR2-KO mice were detected in the severity of pancreatitis caused by 6-hourly repeated administration of caerulein in the present study, although the latter mice showed more profound abdominal allodynia/hyperalgesia than the former. This is inconsistent with an independent work showing that 10-hourly repeated doses of caerulein caused more profound pancreatitis in PAR2-KO mice, as compared with WT animals (Sharma *et al*., 2005). It is likely that PAR2 activation by endogenous agonist proteinases plays anti-inflammatory roles in severe, but not relatively mild, pancreatitis models. In this context, the potential antinociceptive property of PAR2 activation during relatively mild pancreatitis, shown in the present study, could be independent of its anti-inflammatory nature, although the exact antinociceptive mechanisms are still open to question. It is likely that PAR2 present in neurons/cells other than C-fiber neurons may be responsible for potential suppression of pancreatitis-related allodynia/hyperalgesia, considering the previous findings that activation of PAR2 present in the C-fiber terminal triggered somatic and visceral pain/hyperalgesia. Immunohistochemical studies have shown that PAR2 is abundantly expressed in acinar, ductal and vascular endothelial cells in the pancreas (Nguyen *et al*., 1999; Kawabata, 2002; Kawabata *et al*., 2002b; Maeda *et al*., 2005). We speculate that PAR2 activation in non-neuronal cells might cause release of antinociceptive cytokines, such as interleukin (IL)-4, IL-10 and IL-13 (Vale *et al*., 2003), leading suppression of pancreatitis-related allodynia/hyperalgesia, since PAR2 is actually capable of enhancing release of various cytokines including IL-4 and IL-13 in epithelial cells and/or monocytes (Colognato *et al*., 2003; Ebeling *et al*., 2005). The mechanisms of PAR2-mediated protection against pancreatitis appear to involve neither endogenous NO nor capsaicin-sensitive sensory nerves, on the basis of the lack of effects of L-NAME and capsaicin treatment on the PAR2-mediated improvement of increased plasma amylase activity in the present study. Of note is that we could not examine effects of L-NAME and capsaicin treatment on PAR2-mediated antinociception during pancreatitis, because either treatment itself exhibited antinociceptive activity (data not shown). Although inhibition of ERK translocation to the nucleus by PAR2 activation *in vivo* has been described in the previous study (Sharma *et al*., 2005),

more direct in-depth analysis would be necessary to fully understand antinociceptive and anti-inflammatory roles of pancreatic PAR2 during pancreatitis.

In summary, PAR2 appears to potentially protect against pancreatitis-related abdominal mechanical allodynia/hyperalgesia in mice. The roles for PAR2 in pancreatic nociceptive processing might be different in the absence and presence of inflammation.

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