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Proteinase-activated receptor-4 evoked colorectal analgesia in mice: an endogenously activated feed-back loop in visceral inflammatory pain

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Abstract

Background *Activation of proteinase-activated receptor-4 (PAR-4) from the colonic lumen has an antinociceptive effect to colorectal distension (CRD) in mice in basal conditions. We aimed to determine the functional localization of the responsible receptors and to test their role in two different hyperalgesia models.* ***Methods*** *Mice received PAR-4 activating peptide (PAR-4-AP, AYPGKF-NH$_2$) or vehicle intraperitoneally (IP), and abdominal EMG response to CRD was measured. The next group received PAR-4-AP intracolonically (IC) with or without 2,4,6-triaminopyrimidine, a chemical tight junction blocker, before CRD. The SCID mice were used to test the role of lymphocytes in the antihyperalgesic effect. The effects of PAR-4-AP and PAR-4-antagonist (P4pal-10) were evaluated in water avoidance stress (WAS) model and low grade 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis. Spinal Fos protein expression was visualized by immunohistochemistry.* ***Key Results*** *The antinociceptive effect of PAR-4-AP disappeared when was administrered IP, or with the blockade of colonic epithelial tight junctions, suggesting that PAR-4-AP needs to reach directly the nerve terminals in the colon. The CRD-induced spinal Fos overexpression was reduced by 43% by PAR-4-AP. The PAR-4-AP was antihyperalgesic in both hyperalgesia models and in mice with impaired lymphocytes. The PAR-4-antagonist significantly increased the TNBS, but not the WAS-induced colonic hyperalgesia.* ***Conclusions & Inferences*** *The antinociceptive effect of PAR-4-AP depends on its penetration to the colonic mucosa. The PAR-4 activation is endogenously involved as a feedback loop to attenuate inflammatory colonic hyperalgesia to CRD.*

Keywords *proteinase-activated receptor, TNBS, visceral pain.*

Abbreviations: Cat-G cathepsin G; CRD colorectal distension; DRG dorsal root ganglion; IBS-D diarrhea-predominant irritable bowel syndrome; IC intracolonically; IP intraperitoneally; MPO myeloperoxidase; P4pal-10 N-palmitoyl-SGRRYGHALR-NH2; PAR-4 proteinase-activated receptor-4; PAR-4-AP PAR-activating peptide; SC subcutaneous; SCID severe combined immunodeficiency; TAP 2,4,6-triaminopyrimidine; TNBS 2,4,6-trinitrobenzene sulfonic acid; WAS water avoidance stress.

INTRODUCTION

Proteinase-activated receptors (PARs), a family of 7-transmembrane G-protein-coupled receptors are activated by the cleavage of their N-terminal domain by serine-proteases, which unmasks a new amino terminal sequence activating the receptor itself.[1] The PARs other than PAR-3 can be cleaved by synthetic peptides, called PAR-activating peptides (PAR-APs), with sequences based on their tethered ligand. The molecule AYPGKF-NH$_2$ is a PAR-4-AP, which has no effect on either PAR-1 or PAR-2,[1] and whose effects are blocked by a PAR-4 antagonist.[2]

The PARs play an important role in gastrointestinal physiology and pathophysiology [3] and are

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involved in the sensation of visceral pain. The PAR-2 activation induces colorectal hypersensitivity to distension in rats,[4] and its activation by high level of serine-proteases is likely to contribute to the pathogenesis of diarrhea-predominant irritable bowel syndrome (IBS-D). Indeed fecal supernatants from IBS-D patients induce colorectal hypersensitivity to distension in mice via PAR-2 activation.[5] Unexpectedly, fecal supernatants from ulcerative colitis patients, containing similar elevated level of serine-proteases as IBS-D patients decrease colorectal sensitivity in the same model through a predominant activation of PAR-4, overriding PAR-2 activation.[6] Further, cathepsin-G (Cat-G), a PAR-4 activator contained in neutrophil granulocytes is highly present in this fecal supernatant, and was identified as promoting such colonic hyposensitivity.[6] Despite its direct proinflammatory properties in the gut associated with increased colonic permeability,[7] luminal activation of colonic PAR-4 by its agonist peptide PAR-4-AP produces similar visceral analgesia to colorectal distension (CRD) in mice.[6] The PAR-4-AP has been detected on the colonic epithelium,[8] but also in dorsal root ganglion (DRG) neurons[9] or immunocytes.[10] Therefore, the localization of the receptors participating in the antinociceptive effect is not clear. Hence, we have first tested if intraperitoneal (IP) administration of PAR-4-AP is able to mimic the hyposensitivity seen after intracolonic (IC) infusion. Despite the presence of PAR-4 on apical site of colonocytes, due to its small size (<15 kDa) PAR-4-AP may be absorbed through tight junctions to reach mucosal nerve terminals. Consequently, we evaluated if the blockade of colonic epithelial tight junctions may change the effect of PAR-4-AP IC infusion. As a next step, to test if colonic lymphocytes are involved in the mechanism we used SCID mice which bear with functionally defective T and B cells due to a mutation in the Prkdc gene.[11] In our previous study, the activation of colonic PAR-4 was analgesic in mice in basal conditions,[6] nevertheless, in colitis, fecal Cat-G may be in contact with inflamed mucosa, where hyperalgesic neuromodulators are highly present. To evaluate a possible antihyperalgesic effect, PAR-4 agonist and Cat-G were also tested in inflammatory (2,4,6-trinitrobenzene sulfonic acid, TNBS) and non-inflammatory (water avoidance stress, WAS) models of colorectal hypersensitivity to distension. In addition, the use of a selective antagonist in these conditions may allow us to detect a possible physiologic analgesic role of PAR-4, in such conditions.

MATERIALS AND METHODS

Animals

The 8–9 week-old (21–23 g) C57BL/6J wild-type male mice, BALB/cBy Prkdc^{scid} (SCID) mice and their BALB/cBy controls (Janvier, Le Genest St-Isle, France) were used in our study. The animals were kept in polypropylene cages in a temperature-controlled room with a 12-h dark–light cycle, water and standard pellets were provided *ad libitum*. All experimental procedures were approved by both the local Institutional and the Midi Pyrenées Animal Care and Use Committees.

Visceral pain measurement

Under anesthesia (xylasine + ketamine, both 1.2 mg SC in 0.07 mL in 0.9% NaCl) two nickel-chrome electrodes (diameter: 0.08 mm) were implanted in the abdominal external oblique muscle and the third in the abdominal skin as described previously.[6] On the 4–7th postoperative days, colorectal distensions were performed as painful stimuli to evoke abdominal electromyographic response as a sign of visceral sensitivity. The animals were placed in a plastic tunnel, and after 1 h of habituation period, mice were lightly anaesthetized by sodium pentobarbital (1 mg in 0.15 mL 0.9% NaCl, IP), and polyethylene perfusion and distension catheters (Fogarty catheter for artherial embolectomy, 4F, balloon length: 1.1 cm, Edwards Lifesciences, Nijmegen, The Netherlands) were inserted into the colon (the tip of the catheters situated at 3.5 and 2.5 cm from the anus, respectively).[5,6] Colorectal infusions were started when mice recovered completely from anesthesia (e.g. in <60 min). The CRD procedure was performed with volumes progressively increasing in 0.02 mL steps from 0 to 0.10 mL, by injecting physiologic saline to the balloon with a Hamilton syringe (500 μL, Hamilton Company, Bonaduz, Switzerland), each step lasting 10 s with 5 min non-distension periods in-between. During the distension periods, the abdominal muscle electrical activity was recorded and analyzed with Powerlab Chart 5 program from AD instruments. Basal EMG activity was subtracted from the EMG activity registered during the periods of distension.

Elucidation of mechanism of action

Effect of IC vs IP PAR-4 activating peptide on colorectal sensitivity Mice received PAR-4-AP (AYPGKF-NH_2, Sigma, St Quentin Fallavier, France; 100 μg in 0.15 mL 0.9% NaCl) or its vehicle in IP bolus injection or in IC infusion. Visceral sensitivity measurements started 1 h following the end of IC infusion and 15 min after the IP administration.

Effect of the tight junction blocker TAP on PAR-4 activation-induced hyposensitivity Mice received 2,4,6-triaminopyrimidine (TAP, Sigma; total dose: 30 μmol per mouse in 0.1 + 0.15 mL 0.9% NaCl) IC 1 h preceding PAR-4-AP IC infusion (as previously described), followed by a parallel administration of IC TAP. Visceral sensitivity measurements started 1 h following the end of infusion.

SCID mice Male SCID mice and their BALB/cBy controls were operated as C57BL/6J mice, and on the 4th postoperative day mice received IC infusion of 100 μg PAR-4-AP or vehicle as described above. Visceral pain measurements started 1 h following the end of infusion.

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Visceral hypersensitivity models

Water avoidance stress model Modification of a previously described protocol has been used.[12] Briefly, C57BL/6J mice were placed on a 3 × 3 cm platform in a 40 × 40 cm size pool filled with tapwater for 1 h on four consecutive days. The animals who fell into the water were gently dried with a towel and placed back to the platform.

Colonic microinflammation Low-grade colonic inflammation was provoked by IC administration (tip of the catheter 3.5 cm from the anus) of a low dose of TNBS (Sigma, 20 mg kg^{-1} mice) in 40 μL 30% ethanol or 0.9% NaCl in C57BL/6J mice on the 4th postoperative day, as described earlier.[13] Visceral sensitivity measurements were performed 72 h after the administration of TNBS. To assess the low-grade colonic inflammation level, myeloperoxydase activity assays were performed.

Myeloperoxidase activity assay Myeloperoxidase (MPO) activity, a marker of polymorphonuclear neutrophil granules, was assessed in colonic tissues according to previously described techniques (Bradley *et al.*, 1982) and measured to provide an index of neutrophil infiltration and intestinal inflammation. Protein concentration was assessed using BCA protein assay kit (Interchim, Montlucon, France) and MPO activity was expressed as units per g of protein.

Treatments One group of WAS and TNBS mice received IC infusion of 100 μg PAR-4-AP or its vehicle and visceral sensitivity was measured 1 h after the end of infusion. Next group of WAS- and TNBS-treated animals were IC infused by 0.025 UN of Cat-G (Sigma, 0.167 UN mL^{-1} in 0.15 mL 0.9% NaCl) or its vehicle and CRD was performed similarly 1 h after the end of infusion. Another group was treated with 0.75 mg kg^{-1} PAR-4 antagonist (P4pal-10, pepducin, NeoMPS, Strasbourg, France; N-palmitoyl-SGRRYGHALR-NH_2 in 0.15 mL 0.9% NaCl) or its vehicle IP, and the response to CRD was tested 30 min after the injection.

Cat-G activity in the feces Following the fourth water avoidance session in the WAS model or 72 h after the administration of TNBS in the colitis model, fecal samples of mice were collected for Cat-G activity measurement, and fecal supernatants were prepared (0.3 g feces in 4 mL 20 mmol L^{-1} Tris HCl, pH: 8.3). Cat-G activity in the fecal supernatant was measured by an enzymatic assay as described previously using *N*-succinyl-Ala-Ala-Pro-Phe p-Nitroanilide (Sigma).[6]

Fos immunohistochemistry

Mice received IC PAR-4-AP infusion (100 μg in 0.15 mL 0.9% NaCl) or its vehicle, 0.9% NaCl and 1 h later underwent the CRD protocol as described above. A group of mice without treatment and distension served as naive controls. One hour after the completion of the CRD, mice were deeply anesthetized with xylazine-ketamine (both 2 mg IP) and perfused transcardially with 50 mL physiologic saline followed by 50 mL of 4% paraformaldehyde. After fixation, lumbosacral segments (L5-S1) of the spinal cord were dissected and removed, postfixed at +4 °C in 4% buffered paraformaldehyde, incubated in 30% sucrose (24 h, +4 °C), embedded (Tissue Tek medium) and frozen in isopentane at −45 °C. Frozen serial sections (35 μm) were collected in phosphate-buffered saline (PBS), then rinsed twice. Sections were stained for Fos-like immunoreactivity using biotin-avidin-peroxidase complex. Briefly, sections were incubated at room temperature in a blocking solution for 30 min and then incubated with rabbit polyclonal Fos antibody diluted in blocking solution (1 : 10 000; Ab-5, AbCys, Paris). The incubated sections were washed twice and incubated with biotinylated goat anti-rabbit secondary antibody, diluted 1 : 1000 in blocking solution, and then incubated with the avidin-biotin complex (Vectastain Elite kit; Vector Laboratories, Paris, France). Peroxidase activity was revealed using diaminobenzidine as chromogene (DAB substrate kit, Vector Laboratories, France). The presence of Fos immunoreactivity was detected as a dark brown reaction product in cell nuclei under a light microscope (90i Nikon, Nikon France, Champigny-sur-Marne, France). The number of cells containing Fos immunoreactivity was counted in the laminae I-II and the area surrounding the central canal (area X) bilaterally in 16 consecutive sections of the lumbosacral segment of the spinal cord (L5-S1), using Lucia G4.8 software.

Statistical analysis

Results are presented as means ± SEM. Data analysis was performed by using Graphpad Prism software (Graph Pad, La Jolla, CA, USA). For the statistical analysis of MPO results, Student *t*-test was used to compare the two groups (control *vs* TNBS). Data obtained in fecal Cat-G activity and Fos immunohistochemistry measurements were compared by analysis of variance, followed by Tukey *post hoc* test. In the visceral pain experiments, means were calculated for each volume from all values in a group receiving the same treatment, and data were compared by analysis of variance, followed by Tukey *post hoc* test.

RESULTS

Comparative influence of IC *vs* IP administration of PAR-4-AP on colorectal sensitivity to distension

When infused intracolonically prior to distension, PAR-4-AP (100 μg) triggered a hyposensitivity to CRD at the distension volumes from 0.04 to 0.08 mL compared with vehicle infusion ($P < 0.05$). In contrast, when injected intraperitoneally at the same dose, PAR-4-AP had no effect on the response to CRD ($P > 0.05$) compared with vehicle. Indeed, abdominal response to CRD after PAR-4-AP administration was significantly different between IC and IP routes at the same distension volumes ($P < 0.05$; Fig. 1A).

Effects of TAP on PAR-4-AP evoked hyposensitivity

The colorectal infusion of TAP blocked the hyposensitive effect of PAR-4-AP IC infusion (Fig. 1B). The PAR-4-AP alone decreased the sensitivity by 54–33% at distension volumes of 0.04–0.08 mL, respectively ($P < 0.05$). On the contrary, PAR-4-AP + TAP did not affect the sensitivity compared with vehicle ($P > 0.05$).

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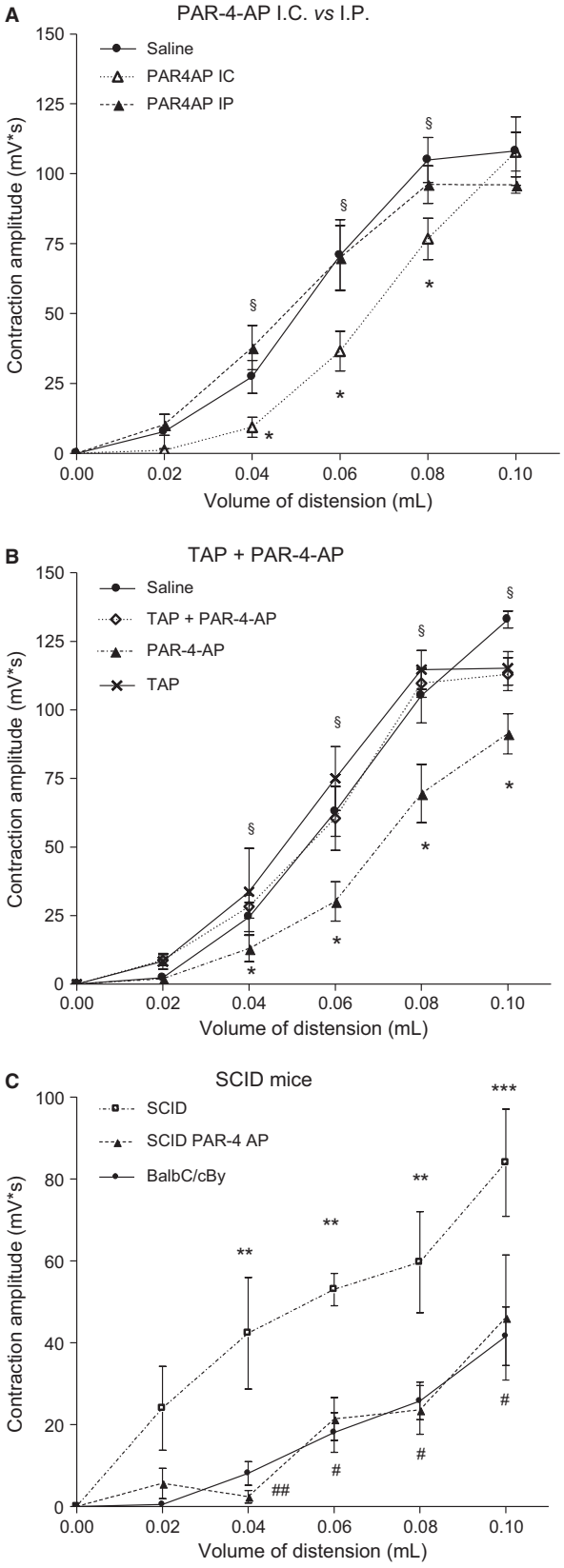

Figure 1 Effect of PAR-4-AP on visceral sensitivity by IP administration, in combination with tight junction blockade or in SCID mice. Electromyographic activity of abdominal muscle contractions evoked by CRD in mice after IP injection or IC infusion of 100 μg PAR-4-AP or its vehicle, 0.9% saline. Each line represents the mean EMG response of all animals tested in the same group. (A) PAR-4-AP was antinociceptive by IC (n = 12, $^{*}P < 0.05$), but not by IP (n = 9, $P > 0.05$) route at the distension levels from 0.04 to 0.08 mL compared with saline (n = 8). The abdominal EMG response was significantly different between IC and IP routes at these distension volumes ($^{\S}P < 0.05$). (B) The blockade of tight junctions by TAP abolished the antinociceptive effect of intracolonic PAR-4-AP (PAR-4-AP, n = 9, *vs* PAR-4-AP + TAP, n = 12; $^{\S}P < 0.05$; whereas PAR-4-AP *vs* saline, n = 8, $^{*}P < 0.05$). The TAP alone had no effect on colorectal sensitivity (TAP, n = 8 *vs* saline, $P > 0.05$). (C) SCID mice (n = 7) were significantly hypersensitive to CRD compared with their BalbC/cBy controls (n = 8; $^{**}P < 0.01$, $^{***}P < 0.001$), which was reversed by PAR-4 activation (n = 8; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$).

The TAP alone had no effect on colorectal sensitivity ($P > 0.05$).

SCID mice

Postoperative complications and mortality was not different in SCID mice compared with their Balb/cBy controls. Compared with their BALB/cBy controls, SCID mice had a significantly greater abdominal response to CRD at the distension levels of 0.04 to 0.1 mL increasing the intensity of EMG response by 384% to 132%, respectively ($P < 0.01$; $P < 0.01$; $P < 0.01$; $P < 0.001$). PAR-4 activation effectively reversed this hypersensitivity ($P < 0.01$, $P < 0.05$; $P < 0.05$; $P < 0.05$; Fig. 1C).

Influence of PAR-4-AP and PAR-4 antagonist on WAS-induced colorectal hypersensitivity

Water avoidance stress significantly increased visceral sensitivity to distension by 730%, 119%, and 69%, at distension volumes of 0.02, 0.04, and 0.06 mL, respectively ($P < 0.01$, $P < 0.05$, $P < 0.05$, Fig. 2A). Intracolonic infusion of PAR-4-AP before distension suppressed the colonic hypersensitivity to distension and restored it to the baseline at all distension volumes. Further, the PAR-4 antagonist had no impact on the visceral hypersensitive response to CRD observed in stressed mice (Fig. 2B).

Influence of PAR-4-AP and PAR-4 antagonist on TNBS-induced colorectal hypersensitivity

Seventy-two hours after intracolonic infusion of TNBS, the abdominal response to CRD was enhanced by 1529%, 98%, and 90% at distension volumes of 0.02, 0.04, and 0.06 mL, respectively, when compared with controls without TNBS ($P < 0.001$, $P < 0.05$, $P < 0.01$, Fig. 3A). Intracolonic infusion of PAR-4-AP, prior to

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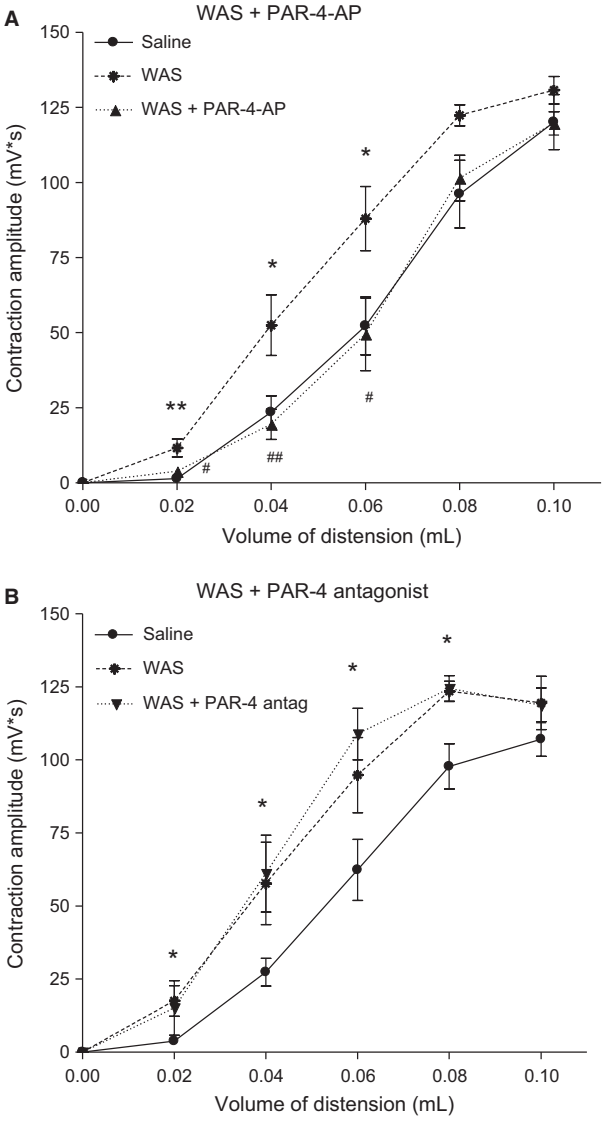

Figure 2 Effect of PAR-4 activation and blockade in water avoidance stress model. Electromyographic activity of abdominal muscle contractions evoked by CRD in mice either 1 h after colorectal infusion of 100 μg PAR-4-AP or its vehicle, or 30 min after PAR-4 antagonist IP injection in water avoidance stress. Each line represents the mean EMG response of all animals tested in the same group. (A) Water avoidance stress increased sensitivity to CRD shown by the elevation in the EMG activity (WAS, $n = 11$ *vs* saline, $n = 10$, $^{*}P < 0.05$, $^{**}P < 0.01$), which was abolished by PAR-4-AP infusion (WAS *vs* WAS + PAR-4-AP; $n = 12$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$). (B) PAR-4 antagonist ($n = 10$) did not change the visceral hypersensitivity provoked by water avoidance stress (saline *vs* WAS, $^{*}P < 0.05$).

distension suppressed the increased sensitivity to CRD for all volumes. Unexpectedly, treatment with the PAR-4 antagonist increased the TNBS-induced hypersensitivity by 78%, 42%, and 19% at distension volumes of 0.04, 0.06, and 0.08 mL compared with TNBS alone ($P < 0.05$, $P < 0.05$, $P < 0.05$; Fig. 3B).

Influence of Cat-G on WAS and TNBS-induced colorectal hypersensitivity

The intracolonic infusion of Cat-G significantly decreased the hypersensitivity observed in WAS-treated mice at volumes from 0.02 to 0.08 mL ($P < 0.05$; $P < 0.05$; $P < 0.01$, $P < 0.01$; Fig. 4A). Surprisingly, Cat-G could not reverse the visceral hypersensitivity induced by low-grade TNBS inflammation (saline *vs* TNBS + Cat-G; $P < 0.05$ at the distension volumes of 0.04 and 0.06 mL and $P < 0.01$ at the volume of 0.08 mL; Fig. 4B).

Cathepsin-G activity in feces of mice after WAS and TNBS treatment

Cat-G activity was significantly increased in feces of mice with TNBS colitis (17.0 ± 2.6 U mg^{-1} prot) compared with naïve controls (8.8 ± 1.9 U mg^{-1} prot). In contrast, Cat-G activity was not elevated in the feces of stressed mice compared with naïve controls (6.9 ± 2.5 U mg^{-1} prot, $P > 0.05$; Fig. 3C).

Evaluation of colonic inflammation induced by a low dose of TNBS

Three days after intracolonic instillation, a low dose of TNBS (20 mg kg^{-1}) induces a slight, but a significant increase of MPO in TNBS mice compared with control (12.7 ± 2.1 *vs* 8.34 ± 1.2, respectively; Fig. 3D). This slight elevated MPO activity was also associated to a slight mucosal thickening in TNBS mice compared with control and to a slight body weight loss 3 days after intracolonic TNBS instillation (Table 1).

Effects of PAR-4-AP on the CRD-induced spinal Fos expression

In naïve mice, the number of Fos-positive cells in the L5-S1 levels of the spinal cord was 7.4 ± 0.7 per section. This number was markedly increased (4.9-fold) after CRD in the group treated with saline compared with naïve, undistended animals ($P < 0.001$; Fig. 5A, B). However, pretreatment with PAR-4-AP IC infusion reduced the CRD-induced Fos expression increase by 46% in the spinal cord compared with saline ($P < 0.001$; Fig. 5A, B).

DISCUSSION

In addition to our previous data showing the antinociceptive effect of intracolonic PAR-4-AP[6] confirmed by others,[14] this work demonstrates that this effect is

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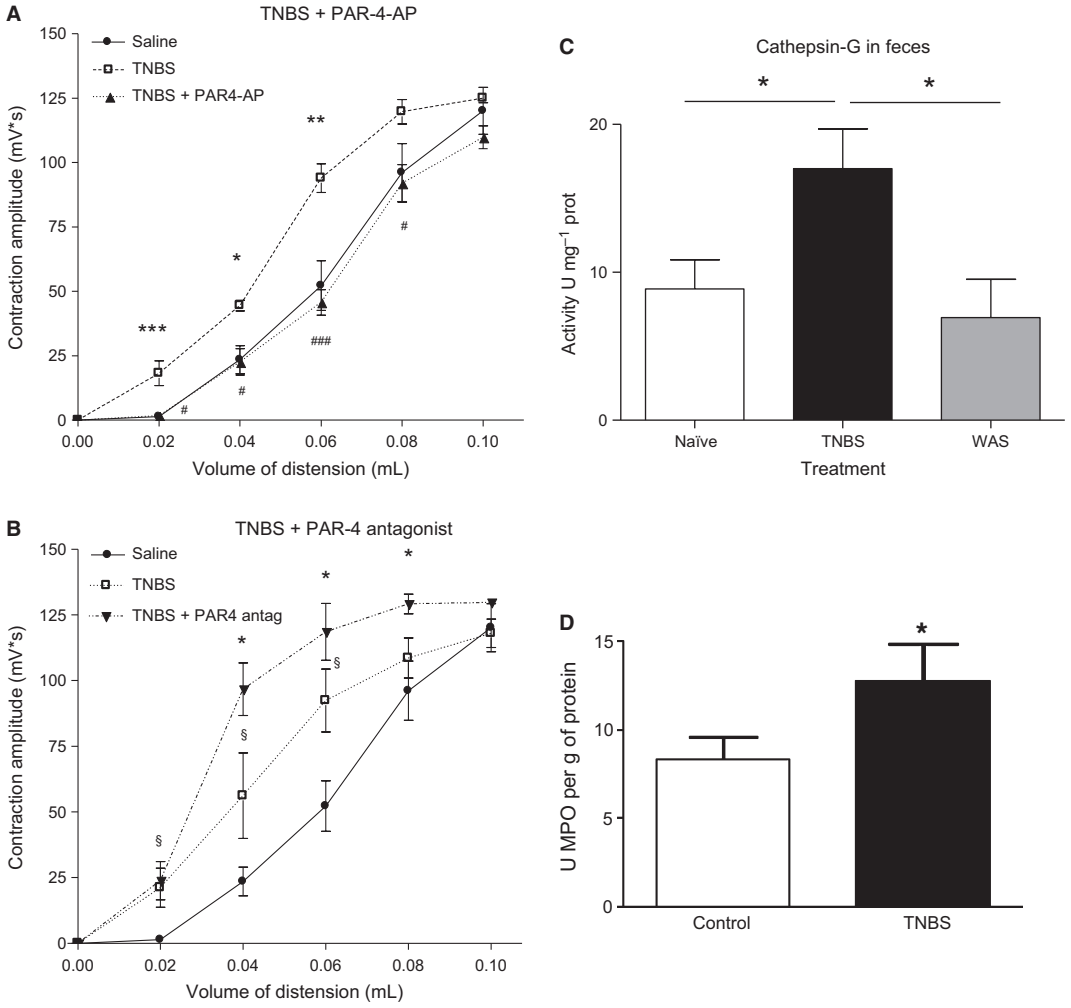

Figure 3 Effect of PAR-4 activation and blockade in TNBS-induced hypersensitivity. Electromyographic activity of abdominal muscle contractions evoked by CRD in mice either 1 h after colorectal infusion of 100 μg PAR-4-AP or its vehicle, or 30 min after PAR-4 antagonist IP injection in TNBS-induced hypersensitivity. Each line represents the mean EMG response of all animals tested in the same group. (A) 72 h after TNBS IC infusion, EMG activity evoked by distension was increased at distension volumes from 0.02 to 0.06 mL, respectively demonstrating higher colorectal sensitivity (TNBS, $n = 12$ *vs* saline, $n = 10$, $^{*}P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.01$). PAR-4-AP infusion reversed the effect (TNBS *vs* TNBS + PAR-4-AP, $n = 11$, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$). (B) Interestingly, the PAR-4 antagonist ($n = 9$) augmented the hypersensitivity compared to TNBS alone (TNBS *vs* TNBS + PAR-4-antagonist $^{*}P < 0.05$, whereas saline *vs* TNBS, $^{\S}P < 0.05$). (C) Cat-G activity significantly increased in feces of mice with TNBS colitis ($n = 8$, $^{*}P < 0.05$) compared with naïve controls ($n = 8$) or WAS-treated animals ($n = 10$). There was no difference between naïve controls and stressed animals ($P > 0.05$). D) MPO activity significantly increased in the colon of mice with TNBS colitis ($n = 10$, $P < 0.05$) compared with naïve controls ($n = 10$).

driven locally. Further, we provide new information on the presence of an endogenous activation of PAR-4 in inflammatory mediated hypersensitivity acting as a feed-back mechanism controlling pain, not activated in stress-induced visceral hyperalgesia.

The localization of PAR-4 responsible for the antinociceptive effect

PAR-4 is present in the colonic epithelium,[8] but also in dorsal root ganglion (DRG) neurons, where its activation does not induce a calcium signal, but reduces the calcium signal evoked by KCl.[9] The involvement of PAR-4 in nociception has been shown in rat knee joint primary afferents as well.[15] Further, patch clamp recordings show that PAR-4 activation in DRG neurons projecting to the colon suppresses their excitability.[16] PARs are also found on the surface of different immune cells throughout the gastrointestinal tract, where their activation may trigger the release of various chemokines and growth factors,[10] interfering with nociceptive pathways. Our present findings on the failure of systemic PAR-4-AP administration to reduce visceral pain sensation is in accordance with

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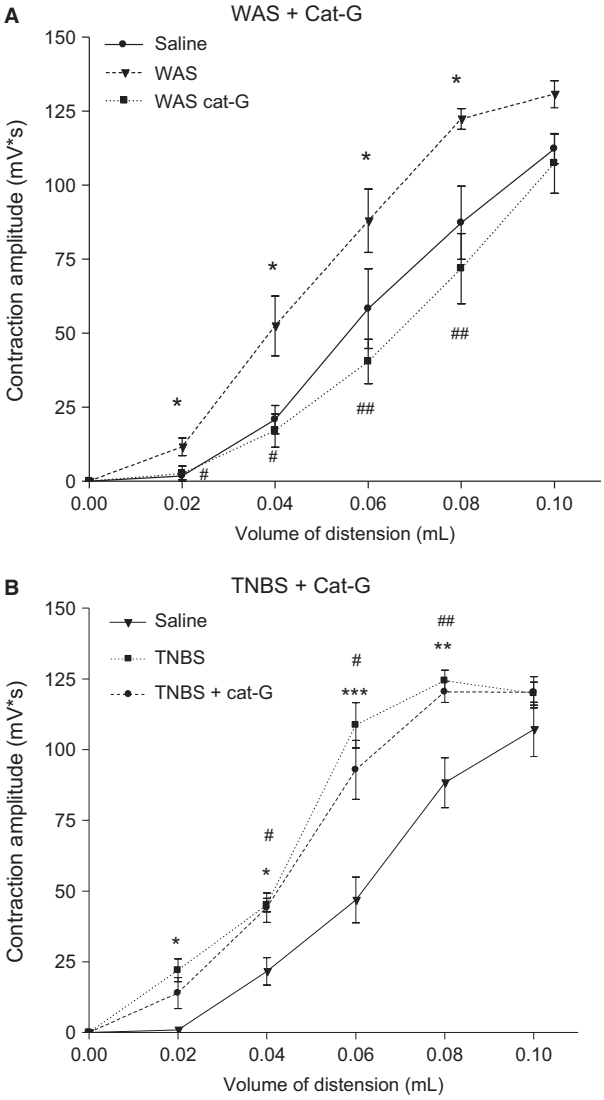

Figure 4 Effect of Cat-G in water avoidance stress and TNBS-induced colorectal hypersensitivity. Electromyographic activity of abdominal muscle contractions evoked by CRD in mice 1 h after colorectal infusion of 0.025 UN of Cat-G or its vehicle. (A) Cat-G restored the basal EMG response in the WAS-induced visceral hypersensitivity demonstrating an antihyperalgesic effect (saline *vs* WAS, $^{*}P < 0.05$; WAS *vs* WAS + Cat-G, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$). (B) In the TNBS model, Cat-G could not influence the visceral hypersensitivity (saline *vs* TNBS, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; saline *vs* TNBS + Cat-G, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

previous data demonstrating that intraplantar injection of PAR-4-AP was antinociceptive in the injected paw, but not in the contralateral paw, showing a local effect.[9] In addition, TAP, a tight junction blocker used for decades not only to block cation-selective permeability in various epithelial tissues[17,18] but also is described as an inotropic agent which acts on potassium conductance in epithelial cell levels, suppresses the antinociceptive effect of PAR-4-AP. TAP activity is preferentially mediated directly by its binding to sites inside the tight junction cation channel.[17] However, there are no *in vivo* studies available comparing the tight junction blocker properties of TAP and its ability to act as an inotropic agent of potassium conductance in epithelial cells. In addition, using TAP agent, a clear link between epithelial intestinal permeability impairment and visceral pain development was described in a rat model of acute stress.[19] This positive correlation is now well established in IBS patients with hypersensitivity, as high visceral pain criterion in these patients is positively correlated with increased intestinal permeability.[20,21] Therefore, taken together, these data suggest that the responsible receptors explaining the antinociceptive effect of PAR-4-AP are located beyond the epithelial cell layer. Nevertheless, the PAR-4-AP elicited increase in colonic permeability[7] is not associated with an increase, but a decrease of nociception, still, the increase of permeability does not rule out the antinociceptive effect.

The PAR-4 is expressed by Kupffer cells, B-lymphocytes,[22] and mast cells,[23] and its activation affects leukocyte mobility.[24,25] Our results show that colonic lymphocytes do not play a role in the antinociceptive effect of PAR-4-AP. Indeed, SCID mice were found hyperalgesic to CRD[26] and reconstitution of the mice with CD4 + T cells restored visceral pain sensation within the normal limits. The antinociceptive effect of PAR-4-AP is still present in SCID mice suggesting that such action does not require functional B or T cells. However, the involvement of other immune cells, like macrophages cannot be excluded with the use of SCID mice, indeed, PAR-4 has been described on the macrophages of rats.[27] Incidentally, we have observed that Balb/cBy mice are hyposensitive in our model of CRD compared with C57BL/6J mice. Strain differences has been described earlier in different experimental models in terms of susceptibility to colitis[28,29] or in antinociceptive responsiveness to N_2O.[30] These data suggest the presence of anatomic and/or functional differences in the immune and neurologic system of these mice strains, which may explain the observed dissimilarity in their response to colorectal distension.

Induction of Fos expression is a marker of neuronal activation, and CRD is a well-known stimulus to activate primary visceral sensory afferents[31] projecting to the lumbosacral spinal cord in mice (L5-S1).[32] CRD induces Fos expression bilaterally in the L6-S2 spinal segments particularly in laminae I and II located in the dorsal horn. We observed a Fos protein overexpression in mice after CRD in the L5-S1 segments of the spinal cord. Parallel to the decreased sensitivity to CRD,

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Table 1 Low-grade inflammatory effect of 20 mg kg^{-1} TNBS in 30% ethanol intracolonically infused in C57BL/6J mice

	Control mice (day 0)	Control mice (day 3)	TNBS mice before TNBS instillation (day 0)	TNBS mice after TNBS instillation (day 3)
Body weight (g)	22.7 ± 0.25	22.9 ± 0.35	23.3 ± 0.18	22.9 ± 0.22#
Colonic length (cm)	–	7.93 ± 0.20	–	7.8 ± 0.08
Macroscopic damage score (colonic mucosal thickening)	–	0 ± 0	–	0.5 ± 0.02*

TNBS, 2,4,6-trinitrobenzene sulfonic acid.
Note only a slight colonic mucosal thickening compared with control and also a slight body weight loss of mice 3 days after TNBS instillation.
Mean ± SEM (n = 10). *P < 0.05 from control mice (day 3); #P < 0.05 from mice before TNBS instillation (day 0).

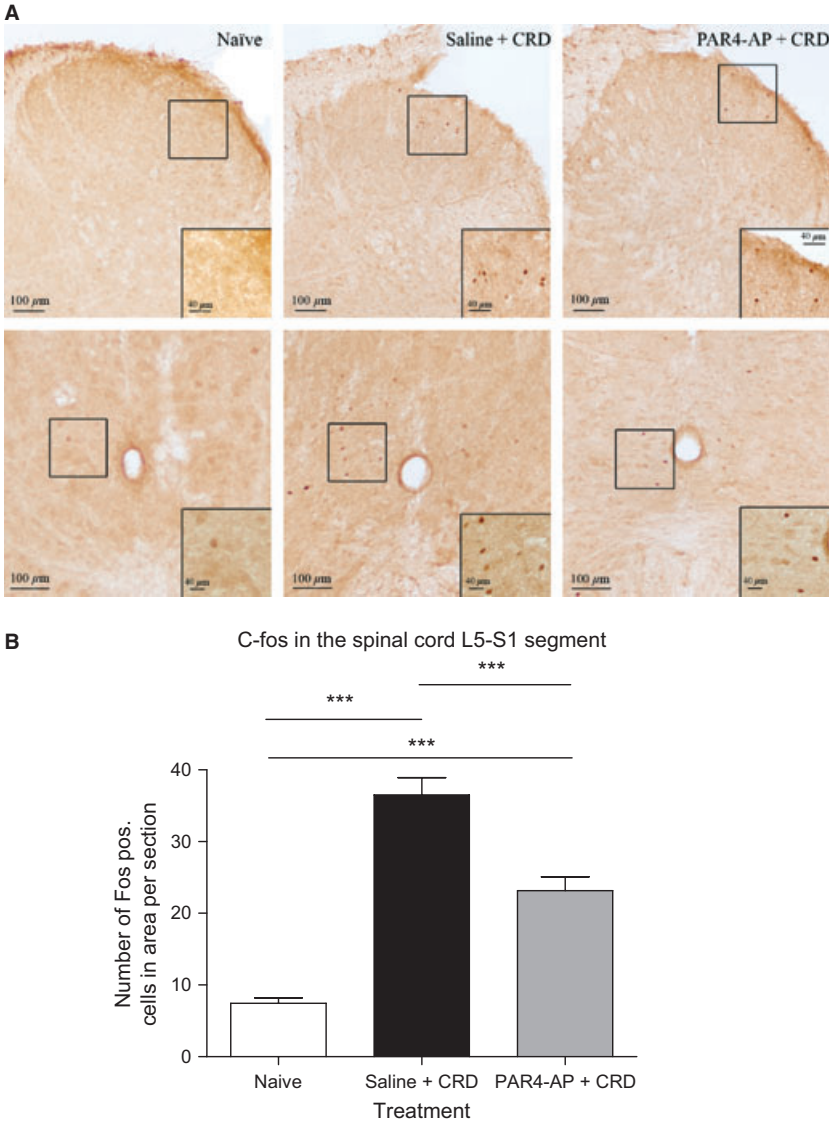

Figure 5 Effect of PAR-4 activation on CRD-induced Fos expression in the spinal cord. (A) Fos immunostaining (arrowheads) of dorsal horn and central area of the spinal cord (segment L5) in naive, undistended animals (n = 5), and after CRD in vehicle (n = 5) or PAR-4 AP-treated (n = 5) mice. (B) Fos expression increased significantly in the lumbosacral spinal cord after CRD in the group treated with 0.9% saline (P < 0.001), whereas PAR-4-AP decreased this CRD-induced Fos expression (P < 0.001).

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PAR-4-AP attenuates Fos expression in the lumbosacral spinal cord. This finding confirms that a decreased neuronal activation is present behind the observed hypoalgesia to CRD.

Endogenous antinociceptive role of PAR-4 in inflammatory, but not non-inflammatory visceral hyperalgesia

The PAR-1 and PAR-4 activation exert analgesic effect to thermal and mechanical stimuli in a rat somatic pain model in both basal and inflammatory conditions.[9,33] Further, PAR-4 activation has been shown to reverse PAR-2 or TRPV-4 activation-induced colorectal hypersensitivity in mice.[14] Herein, we have extended these observations to two visceral hypersensitivity models. Stress is followed by the activation of colonic mast cells[34] responsible for a colonic barrier dysfunction.[35] In contrast, in inflammatory hyperalgesia (colitis) model, inflammatory mediators, such as bradykinin can participate to the higher response to colonic distension.[36] Our results demonstrate that despite the different origins of colorectal hyperalgesia, PAR-4-AP exerts similar antihyperalgesic effects.

Surprisingly, in the TNBS colitis model the colorectal hyperalgesia was augmented by blocking PAR-4 with an antagonist. This suggests the presence of an endogenous activation of PAR-4 in colonic inflammation, exerting a feed-back antinociceptive effect, which was not observed in the stress model. One explanation may be that in TNBS colitis an invasion of neutrophil granulocytes appears in the colonic mucosa, acting as a source of inflammatory mediators, such as Cat-G, a potent activator of PAR-4[37] that reach sensitive nerve terminals and cause hyposensitivity to CRD.[6] In agreement with such hypothesis, using a substrate not completely specific, but largely used as one of the most specific substrate for Cat-G activity evaluation,[38,39] we found an increase of Cat-G activity in the feces associated with neutrophil infiltration in mice with low-grade TNBS colitis, but not in that of stressed mice. Taken together these data may highlight a new role of Cat-G as an endogenous PAR-4 agonist activating a feed-back loop to decrease pain in inflammatory conditions. According to the literature, we can also hypothesize that PAR-4 activated by Cat-G can exert this physiologic antinociceptive feedback loop similar to PAR-1 in inflammatory condition via the release of endogenous mediators like opioids. Indeed, PAR-1 activation modulate paw inflammatory pain by triggering the production of proenkephalin and the activation of opioid receptors.[40]

Besides, the intracolonic administration of Cat-G reversed the colorectal hypersensitivity in mice evoked by WAS, but not by TNBS, whereas PAR-4-AP was antihyperalgesic in both models. This finding can be explained by the non-specificity of Cat-G on PAR-4. Thus, Cat-G may disarm PAR-1 and PAR-2 on human bronchial fibroblasts.[41] Further, in cardiomyocytes, Cat-G induces apoptosis *via* a PAR-independent mechanism.[37] On the other hand, inflammatory mediators released in specific conditions may alter the expression of PARs, which may affect PAR signaling locally.[41,42] Namely, TNF-α and LPS upregulates PAR-2 and induces PAR-4 mRNA expression in human bronchial fibroblasts.[41] We may hypothesize that similar changes appear in TNBS-induced colonic inflammation, which alters the effects of Cat-G on PARs due to its non-specificity. Indeed, in the WAS model, where Cat-G release to the colonic wall is not relevant, the endogenous PAR-4 activation is not detectable, and extrinsic Cat-G exhibits an analgesic effect by PAR-4 activation, similar to PAR-4-AP. However, in the TNBS model, endogenous Cat-G is already highly present in the colonic wall, and activates PAR-4 to exert an analgesic effect. Although PAR-4-AP, a pure PAR-4 agonist is still antinociceptive in this model, additional extrinsic Cat-G does not reinforce the analgesic effect. This observation highlights the natural antinociceptive role of Cat-G in endogenous concentrations in the TNBS model, whereas increasing the concentration by external administration brings the non-specific effects of Cat-G to the front.

CONCLUSIONS

In summary, our results indicate that the antinociceptive effects of PAR-4-AP are linked to a direct or indirect activation of receptors located on colonic nerve terminals, and that PAR-4-AP administration is antinociceptive not only in basal, but also in stress and inflammatory conditions. Endogenous activation of PAR-4 plays an antinociceptive role in mild-TNBS colitis, but not in stress-induced visceral hyperalgesia, and Cat-G is a possible candidate to activate this endogenous antinociceptive mechanism.

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AUTHOR CONTRIBUTION

AA performed the research, analyzed the data and wrote the article. MD, CSC, and AP performed the research and prepared the figures. GK, AR, RR helped in the design of the study and the writing of the article. VT, TW, LB designed the study. HE designed the study, performed the research, analyzed the data and wrote the article.

COMPETING INTERESTS

None.

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