Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Protective Effect of Proteinase-Activated Receptor 2 Activation on Motility Impairment and Tissue Damage Induced by Intestinal Ischemia/Reperfusion in Rodents

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We hypothesized that proteinase-activated receptor-2 (PAR₂) modulates intestinal injuries induced by ischemia/reperfusion. Ischemia (1 hour) plus reperfusion (6 hours) significantly delayed gastrointestinal transit (GIT) compared with sham operation. Intraduodenal injection of PAR₂-activating peptide SLIGRL-NH₂ significantly accelerated transit in ischemia/reperfusion but not in sham-operated rats. GIT was significantly delayed in ischemia/reperfusion and sham-operated PAR₂^{-/-} mice compared with PAR₂^{+/+}. SLIGRL-NH₂ significantly accelerated transit in ischemia/reperfusion in PAR₂^{+/+} but not in PAR₂^{-/-} mice. Prevention of mast cell degranulation with cromolyn, ablation of visceral afferents with capsaicin, and antagonism of calcitonin gene-related peptide (CGRP) and neurokinin-1 receptors with CGRP₈₋₃₇ and RP67580, respectively, abolished the SLIGRL-NH₂-induced stimulatory effect on transit in ischemia/reperfusion. Tissue damage was significantly reduced by SLIGRL-NH₂; this effect was not observed in cromolyn-, capsaicin-, or RP67580-treated rats but was detected following CGRP₈₋₃₇. Intestinal PAR₂ mRNA levels were not affected by SLIGRL-NH₂ in ischemia/reperfusion. We propose that PAR₂ modulates GIT and tissue damage in intestinal ischemia/reperfusion by a mechanism dependent on mast cells and visceral afferents.

PAR₂ effect on transit might be mediated by CGRP and substance P, whereas the effect on tissue damage appears to involve substance P but not CGRP. PAR₂ might be a signaling system in the neuroimmune communication in intestinal ischemia/reperfusion. (Am J Pathol 2006, 169:177–188; DOI: 10.2353/ajpath.2006.051098)

Acute intestinal ischemia is a life-threatening gastrointestinal emergency that remains a major clinical problem with a high mortality rate (~70%).¹ Intestinal ischemia occurs in a wide variety of clinical manifestations, including mesenteric vascular occlusion, neonatal necrotizing enterocolitis, abdominal angina, and Crohn's disease.^{2–9} Graft ischemia is also a serious complication of small bowel transplantation.^{10,11} Ischemic injury due to severe loss in intestinal blood flow can result in many clinical consequences ranging from bleeding, intestinal perforation, and peritonitis to more serious systemic conditions, including myocardial and renal failure, sepsis, multiple organ dysfunction syndrome, and death.¹² Intestinal ischemia and reperfusion induce an acute inflammatory response that is associated with enhanced generation and release of proteinases from different sources, including inflammatory cells, like mast cells and neutrophils, and the coagulation cascade, in addition to digestive and bacterial proteinases normally present in the lumen.^{13–17} Furthermore, breakdown of the gut barrier occurs with bacterial translocation¹⁸; thus, luminal digestive and pancreatic proteinases may penetrate through the mucosa and the muscle layers of the intestine. These enzymes

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are potential activators of proteinase-activated receptors (PARs), a family of G-protein-coupled receptors that are activated by proteolytic cleavage within the amino terminus exposing a tethered ligand domain that binds and activates the receptors.¹⁷ Trypsin and mast cell tryptase are considered as the most likely activators of proteinase-activated receptor-2 (PAR₂) in the gut.^{19,20} PAR₂ is abundantly expressed in the gastrointestinal tract, where it is localized to epithelial, endothelial, muscle, neuronal, and immune cells.^{21–23} PAR₂ modulates several gastrointestinal functions, including motility and secretion.^{17,24,25} In addition, PAR₂ agonists have been reported to either have a pro-inflammatory or anti-inflammatory role in intestinal inflammation^{17,26,27} depending on the model system, the time-course administration, and the cell targets.

Intestinal ischemia with reperfusion induces mast cell degranulation that triggers inflammatory infiltrates associated with increased mucosal permeability, thus resulting in mucosal dysfunction.²⁸ In the gut, mast cells are often in close vicinity to visceral afferents that express PAR₂.²³ These observations provided the background for our hypothesis that PAR₂ modulates intestinal injuries induced by intestinal ischemia/reperfusion through the involvement of mast cells and visceral afferents. To test this hypothesis, we used a model of intestinal ischemia developed in rats by reversible occlusion of the superior mesenteric artery for 1 hour followed by 6 hours of reperfusion. This experimental procedure induces transient mucosal damage and alterations of motor activity.^{8,29} The aims of the study were to investigate: 1) whether PAR₂ activation with a selective PAR₂ agonist affects gastrointestinal motility impairment and mucosal damage in rats with intestinal ischemia followed by reperfusion (I/R) compared with sham-operated (SO) mice and in mice with or without deletion of the PAR₂ gene (PAR₂^{−/−} and PAR₂^{+/+}); 2) the expression of PAR₂ mRNA in the intestine of I/R rats following PAR₂ activation; 3) whether the effects of PAR₂ activation on motility and tissue damage in I/R involve mast cells and extrinsic primary afferent nerves; and 4) the role of calcitonin gene-related peptide (CGRP) and substance P (SP), peptides that control inflammation and pain and colocalize with PAR₂ in visceral afferents,^{27,30,31} in mediating PAR₂ effects in intestinal ischemia.

Materials and Methods

Surgical Procedures and PAR₂ Activation

Animal care and procedures were in accordance with the National Institutes of Health recommendations for the humane use of animals. All experimental procedures were reviewed and approved by the appropriate Animal Use Committee of the University of California, Los Angeles and the Veterans Affairs Greater Los Angeles Health System (VAGLAHS, Los Angeles, CA), and the University of Calgary (Calgary, AB, Canada). Male Wistar rats (175 to 200 g) were purchased from Harlan World Headquarters (Indianapolis, IN). C57BL6 mice, either female or male, (20 to 25 g) were obtained from Charles River Laboratories (Montreal, QC, Canada), and PAR₂^{−/−} and

wild-type littermates were obtained from Johnson & Johnson Pharmaceutical Research Institute (Spring House, PA). Animals were fasted with free access to water for 12 to 18 hours before experimental procedures. Rats were anesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL) (40 mg/kg; intraperitoneally). Following abdominal laparotomy, the small bowel was retracted to the right and the superior mesenteric artery was identified. The superior mesenteric artery was occluded just proximal to the right colic artery, causing ischemia to the small bowel for 1 hour. Intestinal ischemia was followed by 6 hours of reperfusion (I/R). This time of reperfusion was chosen to avoid interference of anesthesia with gastrointestinal transit measurement and to allow the development of acute inflammation. SO animals, in which abdominal laparotomy and artery isolation were performed without occlusion of the vessel, served as controls. The survival rate of animals in each experimental group was 100%. To determine the effect of PAR₂ stimulation on intestinal ischemia and reperfusion, the selective PAR₂ agonist SLIGRL-NH₂ or the inactive, scrambled control peptide LRGILS-NH₂ was used. Peptides were synthesized by Byo-Synthesis Inc. (Lewisville, TX) and by the peptide synthesis facility of the University of Calgary. Their concentration, purity, and composition were determined by high performance liquid chromatography, mass spectrometry, and quantitative amino acid analysis. Peptides were administered intraduodenally (3.5 mg/kg in 1 ml/kg) at the beginning of the reperfusion in I/R rats and following surgery in SO animals. The aminopeptidase inhibitor amastatin (1.25 mg/kg, intraduodenally; Sigma-Aldrich, St. Louis, MO) was simultaneously administered to avoid peptide degradation. The midline incision of the abdominal wall was then closed by two-layer sutures. Following recovering from anesthesia, animals were returned to their cages. Similar procedures were used for both the PAR₂^{+/+} and PAR₂^{−/−} mice (C57BL6 strain), except that the superior mesenteric artery was occluded for 30 minutes followed by 6 hours of reperfusion. Animals were euthanized at the end of the experiments by deeply anesthetizing them with an overdose of sodium pentobarbital (50 mg/kg, intraperitoneally) followed by thoracotomy.

Cromolyn Pretreatment

To determine the role of mast cells in intestinal ischemia with and without PAR₂ activation, the mast cell stabilizer cromolyn (20 mg/kg; Sigma-Aldrich)³² was administered through the tail vein 1 hour before the induction of ischemia or sham operation in I/R and SO rats with or without intraduodenal treatment with PAR₂ agonist. All chemicals were dissolved in sterile saline.

Ablation of Extrinsic Visceral Afferent Neurons

To determine the involvement of extrinsic visceral afferents in I/R-induced alterations with and without PAR₂ activation, chemical ablation of extrinsic primary afferent nerves was induced by treatment with the neurotoxin

capsaicin (125 mg/kg; Sigma-Aldrich). The total dose of capsaicin was divided into three injections (25 mg/kg in the morning, 50 mg/kg after 6 hours, and 50 mg/kg after 32 hours) and administered subcutaneously (as an emulsion of 12.5 mg/ml capsaicin in a medium containing 10% ethanol, 10% of Tween 80, and 80% of saline) in rats under isoflurane (0.5 to 3%) anesthesia. Animals were then left for 10 days, and I/R or sham operation was performed with or without intraduodenal administration of PAR₂ agonist. To evaluate the effectiveness of capsaicin pretreatment, we used the eye-wipe response to a diluted (0.01%) capsaicin solution.

Pretreatments with CGRP and SP Receptor Antagonists

To determine the involvement of the neuropeptides CGRP and SP, both coexpressed with PAR₂ by a large proportion of primary sensory visceral afferents,^{27,30,31} in exogenous stimulation of PAR₂ in I/R-induced responses, animals were pretreated with either CGRP receptor antagonist CGRP₈₋₃₇³³⁻³⁵ or RP67580, a selective antagonist for the preferred SP receptor neurokinin-1 (NK₁).³⁶ CGRP₈₋₃₇ was administered at a dose of 150 µg/kg in the tail vein 15 minutes before the induction of ischemia and then every 2 hours (for a total of four injections), because this compound degrades. RP67580 (1 mg/kg) was administered in the tail vein 15 minutes before the ischemia followed by another injection at 0.5 mg/kg 3 hours later.³⁵

Upper Gastrointestinal Transit (GIT)

A nonabsorbable black marker (10% charcoal suspension in 5% gum arabic; Sigma-Aldrich) was administered by gavage to conscious animals 5 hours and 15 minutes after the beginning of reperfusion as described elsewhere with minor modifications.^{25,37} Forty-five minutes later, animals were euthanized with sodium pentobarbital, and the small bowel was removed. The distance traveled by the marker was expressed as a percentage of the total length of the small intestine from pylorus to cecum.

Assessment of Tissue Damage: Microscopic Damage Score

Specimens of the distal ileum were collected from the different groups of animals at the end of the perfusion period to determine the level of tissue damage. Following overnight fixation in 10% formalin, specimens of the ileum were embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin. Microscopic histological damage score was evaluated by a person unaware of the treatments and was based on a semiquantitative scoring system in which the following features were graded: extent of destruction of normal mucosal architecture (0, normal; 1, 2, and 3, mild, moderate, and extensive damage, respectively), presence and degree of cellular infiltration (0, normal; 1, 2, and 3, mild, moderate, and trans-

mural infiltration), extent of muscle thickening (0, normal; 1, 2, and 3, mild, moderate, and extensive thickening), presence or absence of crypt abscesses (0, absent; 1, present), and presence or absence of goblet cell depletion (0, absent; 1, present). The scores for each feature were then summed with a maximum possible score of 11 as previously described.^{27,30,38,39}

SYBR Green Real-Time RT-PCR

PAR₂ mRNA expression was determined by SYBR Green I real-time quantitative polymerase chain reaction (PCR) using an Mx3000P real-time PCR detection system (Stratagene, LA Jolla, CA). Total mRNA was extracted from duodenum and ileum using the absolutely RNA® RT-PCR Miniprep kit (Stratagene) as previously described.⁴⁰ After verification of its integrity, RNA was quantified spectrophotometrically, and 1 µg was processed for complementary DNA (cDNA) synthesis using SuperScript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA). Specific primers for PAR₂ gene were designed by using Primer3 software. The sequences of the primers used were: PAR₂, sense: 5'-AAC ATC ACC ACC TGT CAC GA-3'; antisense: 5'-CAC GTA GGC AGA CGC AGT AA-3'; β-actin, used as a housekeeping gene, sense: 5'-TCA TGA AGT GTG ACG TTG ACA TCC GT-3'; antisense: 5'-CTT AGA AGC ATT TGC GGT GCA CGA TG-3' (Promega Corp., Madison, WI).

The efficiency of the real-time RT-PCR primer pairs was determined by amplifying serial dilutions of cDNA. The real-time RT-PCR was performed using Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen). Each cycle consisted of three steps: denaturation for 30 seconds at 95°C, annealing for 30 seconds at 55°C, and 30 seconds of elongation at 72°C. The data acquired from each sample were normalized to those of β-actin. The specificity of the real-time reverse transcriptase (RT)-PCR was further confirmed by a regular RT-PCR followed by agarose gel electrophoretic analysis to verify the presence of a single band corresponding to the size predicted for the amplicon. Relative quantification was performed by using the comparative cycle threshold method as described elsewhere (User Bulletin #2, ABI Prism 7700 Sequence Detection System, December 11, 1997).

Statistical Analysis

Differences among groups that underwent I/R and SO were analyzed using two-way analysis of variance, followed by the Bonferroni post-test. Differences between treatments within the same experimental conditions (ie, I/R or SO) were evaluated using one-way analysis of variance, followed by Dunnett's post-test. The same test was used to compare differences between mRNA measurements obtained with real-time RT-PCR. Values are expressed as means ± SE. A *P* value of <0.05 was required to consider group differences as significant, and a *P* value of <0.01 was considered highly significant.

Figure 1. This graph shows the effect of PAR₂-selective agonist SLIGRL-NH₂ (with amastatin), saline, control peptide LRGILS-NH₂ (with amastatin), or amastatin alone administered intraduodenally on upper GIT (measured as percent transit of charcoal) in SO (white bars) or I/R (black bars) rats. Upper GIT was significantly delayed in I/R compared with SO rats treated with saline; SLIGRL-NH₂ significantly accelerated GIT in I/R rats, but not in SO animals ($P < 0.001$); LRGILS-NH₂ or amastatin alone did not affect GIT. Data are expressed as mean $\pm$ SE of 8 to 10 animals per group; °Significant difference from the corresponding SO group ($P < 0.05$; I/R-saline vs. SO-saline; $P < 0.001$ I/R-SLIGRL vs. SO-SLIGRL); *Significant difference from the corresponding saline group ($P < 0.001$).

Results

SLIGRL-NH₂ Reverses Ischemia-Induced GIT Delay in Rats

Gastrointestinal transit was significantly delayed in I/R compared with SO rats (42.7 ± 3.6 vs. 56.8 ± 3.2 , $P < 0.05$) (Figure 1). Intraduodenal administration of SLIGRL-NH₂ with amastatin significantly accelerated the gastrointestinal transit in I/R rats (77 ± 3.9 vs. 42.7 ± 3.6 , $P < 0.001$) but not in SO animals, indicating a stimulatory role of PAR₂ on motility in ischemic conditions. The inactive control peptide LRGILS-NH₂ with amastatin or amastatin alone did not affect GIT in either I/R or SO rats, supporting the specificity of the effect detected.

SLIGRL-NH₂ Reverses Ischemia-Induced GIT Delay in PAR₂^{+/+} but Not in PAR₂^{-/-} Mice

In PAR₂^{-/-} mice, there was a significant delay in GIT in both I/R and SO animals without PAR₂ agonist pretreatment, compared with the wild-type groups (I/R: 26.1 ± 2.3 vs. 38.5 ± 1.9 , $P < 0.001$; SO 37.2 ± 2.6 vs. 56.9 ± 1.6 , $P < 0.001$) (Figure 2). These data suggest that endogenous activation of PAR₂ is involved in the regulation of gastrointestinal transit under normal conditions and potentially under ischemic conditions as well. Treatment with SLIGRL-NH₂ significantly accelerated gastrointestinal transit in I/R PAR₂^{+/+} (60.0 ± 5.1 vs. 38.5 ± 1.8 , $P < 0.001$) but not in PAR₂^{-/-} mice, further supporting the specificity of PAR₂ agonist treatment in PAR₂^{+/+}.

Figure 2. Effects of PAR₂-selective agonist SLIGRL-NH₂ on GIT in PAR₂^{+/+} and PAR₂^{-/-} mice. GIT was significantly delayed in PAR₂^{+/+} and PAR₂^{-/-} ischemic mice (black bars) compared with PAR₂^{+/+} and PAR₂^{-/-} sham operated animals (white bars) treated with saline ($P < 0.01$). Treatment with SLIGRL-NH₂ (with amastatin) significantly accelerated GIT in I/R PAR₂^{+/+} ($P < 0.001$) but not in PAR₂^{-/-} mice, further supporting the specificity of PAR₂ agonist treatment. Data are expressed as mean $\pm$ SE of 7 to 8 animals per group. °Significant difference from the corresponding SO group ($P < 0.01$); *Significant difference from the corresponding saline group ($P < 0.05$ PAR₂^{-/-} I/R-saline; $P < 0.01$ PAR₂^{-/-} SO-saline and PAR₂^{-/-} I/R-SLIGRL); *Significant difference from the corresponding saline group.

Figure 3. Effects of PAR₂-selective agonist SLIGRL-NH₂ on GIT in cromolyn-pretreated rats. Pretreatment with cromolyn did not affect the GIT in ischemic (I/R; **black bars**) and sham-operated (SO; **white bars**) rats but prevented the acceleration induced by SLIGRL-NH₂ (with amastatin) in I/R rats. Data are expressed as mean $\pm$ SE of 6 to 8 animals per group. [°]Significant difference from corresponding SO group ($P < 0.001$); ^{*}Significant difference from the corresponding group without cromolyn pretreatment ($P < 0.001$); [#]Significant difference from the corresponding saline group.

Mast Cells and Visceral Afferent Neurons Mediate the Accelerating Effect of PAR₂ Agonist on GIT in I/R

Pretreatment with cromolyn did not significantly affect the GIT in I/R and SO rats but prevented the acceleration induced by SLIGRL-NH₂ in I/R rats (51.7 ± 2.6 in cromolyn treated vs. 77.0 ± 3.9 in non-cromolyn treated; $P < 0.001$) (Figure 3). This suggests that in I/R rats PAR₂ activation mediates the increase in GIT through degranulation of mast cells.

Chronic capsaicin pretreatment induced ablation of visceral afferent neurons as indicated by the lack of eye wiping following administration of a drop of diluted capsaicin in capsaicin-treated rats. In animals treated with the PAR₂-activating peptide, chronic capsaicin significantly reduced GIT in SO ($P < 0.01$ vs. SO-saline) and prevented the accelerating effect of SLIGRL-NH₂ in I/R rats (36.8 ± 1.3 in capsaicin-treated vs. 77.0 ± 3.9 in non-capsaicin-treated; $P < 0.001$) (Figure 4). This inhib-

itory effect suggests that visceral afferents participate to the PAR₂ action on GIT in intestinal ischemia with reperfusion. In addition, in capsaicin-treated animals, I/R did not induce a significant delay in GIT as compared to SO animals, suggesting a role of visceral afferent neurons in the control of the gastrointestinal motility in intestinal ischemia followed by reperfusion.

Effect of CGRP and NK₁ Receptor Blockade on PAR₂-Mediated Changes in GIT in I/R

Antagonism of CGRP receptor by pretreatment with CGRP₈₋₃₇ or antagonism of NK₁ receptor by RP67580 prevented the SLIGRL-NH₂-induced increase in GIT in I/R rats (38.4 ± 8.9 in CGRP₈₋₃₇-treated and 43.1 ± 6.0 in RP67580-treated vs. 77.0 ± 3.9 in non-treated; $P < 0.001$) (Figures 5). CGRP and NK₁ receptor blockade did not significantly modify the GIT in I/R and SO animals in the absence of SLIGRL-NH₂ (see Figure 5; compare to Figure

Figure 4. Effects of PAR₂-selective agonist SLIGRL-NH₂ on GIT in capsaicin-pretreated rats. There were no differences in transit among the different groups treated with capsaicin. Ablation of extrinsic primary afferent nerves prevented the acceleration induced by SLIGRL-NH₂ (with amastatin) observed in ischemic rats (**black bars**) not treated with capsaicin. Data are expressed as mean $\pm$ SE of 5 to 6 animals per group. ^{*}Significant difference from corresponding SO group; [°]Significant difference from equivalent groups without capsaicin pretreatment ($P < 0.001$ I/R-SLIGRL; $P < 0.01$ SO-SLIGRL) illustrated in the figure. Capsaicin treatment also prevented the GIT delay induced by I/R suggesting a role of visceral afferents in the control of gastrointestinal motility in intestinal ischemia.

Figure 5. Effects of PAR₂-selective agonist SLIGRL-NH₂ on GIT in rats pretreated with CGRP antagonist, CGRP₈₋₃₇, or SP antagonist RP67580 (specific for NK₁ receptors). The pretreatment with CGRP₈₋₃₇ and RP67580 did not modify the GIT in I/R rats (**black bars**), nor in SO group (**white bars**), when compared with correspondent control groups without CGRP or NK₁ blockage (shown in Figure 1). By contrast, CGRP₈₋₃₇ and RP67580 pretreatment completely abolished the stimulatory effect on GIT induced by SLIGRL-NH₂ administration when compared with I/R-SLIGRL rats with no pretreatment. NK₁ receptor blockade prevented the I/R-induced delay of GIT suggesting a role of SP in the control of gastrointestinal motility in intestinal ischemia. Data are expressed as mean $\pm$ SE of 4 to 10 animals per group. *Significant difference from equivalent groups without treatment with any antagonist ($P < 0.001$).

1). However, blockade of either receptor prevented the GIT delay induced by I/R as observed with capsaicin treatment.

Together with the results obtained with chronic capsaicin treatment, these findings suggest that visceral afferent neurons mediate the PAR₂ activation-induced effect on GIT in I/R through the release of CGRP and SP.

SLIGRL-NH₂ Improves Ischemia-Induced Mucosal Damage in Rats

Figure 6 illustrates the total microscopic damage scores (white bars) and the leukocyte recruitment (gray bars) in each group of rats from the first set of experiments (no pretreatment with cromolyn, capsaicin, or CGRP and NK₁ receptor antagonists). Signs of inflammation were observed in I/R animals treated with saline, control peptide LRGILS-NH₂, or amastatin as indicated by the significant increase in both total microscopic damage score (white

bars; $P < 0.001$) and leukocytes infiltration (gray bars; $P < 0.05$) when compared with the corresponding SO groups. By contrast, in the I/R group that received treatment with SLIGRL-NH₂ (plus amastatin), total microscopic damage was significantly decreased when compared with I/R controls (1.29 ± 0.29 vs. 4.57 ± 0.65 ; $P < 0.001$), whereas leukocyte recruitment was not significantly different. These results suggest that most of the tissue damage induced by ischemia followed by reperfusion is not due to inflammatory cell infiltrates and that the protective effect of PAR₂ exogenous activation on ischemia-induced mucosal damage affects parameters different from leukocyte recruitment. The inactive control peptide LRGILS-NH₂ or amastatin alone did not affect the damage score in I/R rats, further suggesting that the protective effects of SLIGRL-NH₂ are specific of PAR₂ activation. In SO animals, PAR₂-activating peptide SLIGRL-NH₂ caused a significant increase in the inflamma-

Figure 6. Effects of PAR₂-selective agonist SLIGRL-NH₂ on microscopic damage score. Inflammatory response was significantly higher in ischemic compared with SO rats in all groups, except for the SLIGRL-NH₂-treated group. SLIGRL-NH₂ significantly reduced damage score in I/R rats, compared with all control groups (saline, LRGILS-NH₂, or amastatin alone; $P < 0.001$). SLIGRL-NH₂ caused a significant increase in microscopic damage score ($P < 0.05$) in SO animals, suggesting that the intraluminal administration of PAR₂ agonist in naïve animals provokes mucosal damage mainly characterized by leukocyte infiltration. Data are expressed as mean $\pm$ SE of 4 to 6 animals per group. *Significant difference from corresponding SO group; #Significant difference from the corresponding saline group.

Figure 7. Histological damage. Representative histological sections of rat intestine from sham-operated rats (**top left**) or ischemia-reperfusion rats (I/R) after saline, LRGLS-NH₂, or SLIGRL-NH₂ intraluminal administration in non-treated rats, cromolyn-treated (CRO) or capsaicin-treated (CAP) rats. **Double arrows** indicate increased muscle thickness, **arrowheads** indicate inflammatory cell infiltration, and **single arrows** indicate mucosal erosion.

tory response, mostly due to a significant increase in leukocytes infiltration, consistent with previous reports^{27,35} (Figure 6). The major differences between SO and I/R groups consisted of changes to the mucosal architecture, massive cellular infiltration, and severe muscle thickening (see Figure 7). The protective effects of SLIGRL-NH₂ were mostly represented by a reduction of mucosal erosion and by less muscle thickening compared with the I/R rats that did not receive SLIGRL-NH₂ (see Figure 7).

SLIGRL-NH₂ Does Not Improve Ischemia-Induced Mucosal Damage in Cromolyn- and in Capsaicin-Pretreated Rats

Figure 8 shows the microscopic damage scores in cromolyn-pretreated rats. In these rats, the mucosal damage score, but not the level of leukocytes infiltration, was significantly higher in I/R groups than in the SO groups. SLIGRL-NH₂ administration did not cause a significant reduction in the inflammatory response in cromolyn-treated I/R rats compared with I/R rats treated with saline (Figure 8). These data suggest that the protective effect of SLIGRL-NH₂ treatment is partially mediated by mast cell degranulation and release of their mediators.

The microscopic damage scores and leukocytes recruitment in capsaicin-pretreated rats are shown in Figure 9. In animals with visceral afferents ablation, the level of mucosal damage (but not of cell infiltration) in the I/R saline group was significantly higher than in the SO saline group. In capsaicin-treated rats, SLIGRL-NH₂ administration did not cause a significant reduction in either mucosal damage or in leukocytes recruitment in I/R rats compared with saline I/R rats (Figure 9). These observations suggest that sensory afferents are involved in the exogenous PAR₂ activation protective effect in I/R-induced inflammation.

Effect of CGRP and NK₁ Receptor Blockade on PAR₂-Mediated Inflammatory Response in I/R

As shown in Figure 10, antagonism of CGRP receptor or NK₁ receptor did not significantly modify the inflammatory response in I/R saline rats when compared with the corresponding I/R group that was not pretreated with CGRP₈₋₃₇ or RP67580 (as shown in Figure 6). Mucosal damage and cell infiltrates were significantly increased in the I/R saline group compared with the SO group pretreated with CGRP₈₋₃₇ or RP67580. Interestingly, whereas NK₁ receptor antagonism reversed the protective effect of SLIGRL-NH₂ on mucosal damage (but not leukocytes recruitment) in I/R rats, CGRP receptor blockade did not, as indicated by comparable levels of mucosal damage and cell infiltrates in SLIGRL-NH₂ I/R rats with or without CGRP₈₋₃₇ pretreatment (compare Figure 10 with Figure 6).

Taken together, these data indicate that the protective effects of SLIGRL-NH₂ against mucosal damage in I/R rats is partially mediated by primary sensory visceral afferents through an involvement of NK₁ receptors but not of CGRP receptors. A possible protective role of CGRP in the absence of ischemia is suggested by the observation that the microscopic damage score (but not of the leukocytes infiltration) in CGRP₈₋₃₇-pretreated SO animals was threefold higher compared with SO rats that did not receive the pretreatment.

Figure 8. Effects of PAR₂-selective agonist SLIGRL-NH₂ on microscopic damage score in cromolyn-pretreated rats. Microscopic damage was significantly higher in I/R-saline compared with SO-saline treated rats ($P < 0.001$); SLIGRL-NH₂-induced reduction of microscopic damage in I/R rats (as observed in Figure 6) was not observed in rats pretreated with cromolyn ($P < 0.05$). Data are expressed as mean $\pm$ SE of 6 to 8 animals per group. *Significant difference from corresponding SO group; #Significant difference from the corresponding saline group; °Significant difference from equivalent groups without treatment with any antagonist.

PAR₂ mRNA Levels Are Not Modified in Ischemic Rats

To investigate whether an up-regulation of PAR₂ expression could account for the SLIGRL-NH₂-induced effects in I/R rats, we measured the levels of PAR₂ mRNA with real-time RT-PCR in specimens of the duodenum (Figure 11A) and ileum (Figure 11B). Intestinal tissues were excised from rats subjected to ischemia and no reperfusion (I/R₀) and ischemia followed by 6 hours of reperfusion (I/R₆) with and without SLIGRL-NH₂ treatment. As shown in Figure 11 (A and B), there was no significant difference in the levels of PAR₂ mRNAs among groups, suggesting that reperfusion with or without SLIGRL-NH₂ treatment did not affect the baseline PAR₂ mRNA expression in ischemic rats.

Discussion

This study shows that: 1) intestinal ischemia followed by 6 hours of reperfusion induces delay of the upper GIT that was reversed specifically by the PAR₂-activating peptide administration, supporting a stimulatory role for PAR₂ on gastrointestinal motility in I/R; 2) endogenous PAR₂ activation is implicated in modulating GIT and in reducing I/R-induced delay of GIT, because PAR₂-deficient mice showed delay in GIT compared with wild-type mice in addition to more severe delay following I/R; 3) exogenous PAR₂ activation also significantly reduced the mucosal damage but did not affect the leukocyte infiltrates induced by I/R; 4) the stimulatory effects of PAR₂ exogenous activation on GIT in I/R was reversed by cromolyn, capsaicin, and blockade of CGRP and NK₁ receptors; 5)

Figure 9. Effects of PAR₂-selective agonist SLIGRL-NH₂ on microscopic damage score in capsaicin-pretreated rats. In animals with visceral ablation the damage score was significantly different in the I/R group compared with the correspondent SO group ($P < 0.05$) and was not significantly reduced by SLIGRL-NH₂- (with amastatin) treatment. Data are expressed as mean $\pm$ SE of 5 to 6 animals per group. °Significantly different from corresponding SO group; #Significant difference from the corresponding saline group; *Significant difference from equivalent groups without capsaicin pretreatment.

Figure 10. Effects of PAR₂-selective agonist SLIGRL-NH₂ on microscopic damage score in rats pretreated with CGRP receptor antagonist, CGRP₈₋₃₇, or NK₁ receptor antagonist, RP67580. Inflammatory response was significantly higher in I/R saline rats compared to correspondent SO rats. RP67580 but not CGRP₈₋₃₇ pretreatment reversed the anti-inflammatory effect of SLIGRL-NH₂, suggesting the involvement of SP but not CGRP in the mucosal protection sustained by exogenous PAR₂ activation. Data are expressed as mean $\pm$ SE of 4 to 10 animals per group. °Significantly different from corresponding SO group ($P < 0.01$ in CGRP₈₋₃₇-treated groups; $P < 0.001$ in RP67580-treated groups); *Significant difference from equivalent groups without Pretreatment with any antagonist.

the protective effect of exogenous activation of PAR₂ against I/R-induced tissue damage was reversed by cromolyn, capsaicin, and NK₁ receptor antagonist treatment, but not by CGRP receptor blockade; and 6) the levels of PAR₂ mRNA in I/R rats pretreated with PAR₂-activating peptide were similar to those in either I/R₀ (no reperfusion) or I/R rats treated with saline. Taken together, these results provide evidence that PAR₂ activation has a protective role in post-ischemic reperfusion-induced injury in the intestine. Both mast cell and visceral afferents contribute to PAR₂ acceleration of GIT in I/R rats through a mechanism that partly involves CGRP and SP release from visceral afferents. The protective effect of exogenous PAR₂ activation against I/R-induced mucosal damage also appears to be mediated by mast cells and primary sensory nerves through a pathway involving SP, but not CGRP, release.

Intestinal ischemia with reperfusion leads rapidly to a disruption of the mucosal barrier, which normally protects the tissues from luminal content. Loss of mucosal integrity has been implicated in the pathogenesis of the multiple organ dysfunction syndrome, a life-threatening complication of intestinal ischemia, which may be caused by a hyper-inflammatory response and by the loss of autoregulation of the normal inflammatory response.¹⁸ Alterations of gastrointestinal motility,^{8,41,42} which can be partly due to structural changes and neuronal plasticity occurring within the enteric nervous system,^{29,43} may also contribute to the development of bacterial overgrowth with subsequent bacterial translocation. Mast cells degranulation has been recognized as an important factor in mediating mucosal damage and motor alterations in small bowel.^{28,44} There is increasing evidence that PAR₂ plays an important role in inflammation acting either as a pro- or anti-inflammatory agent, depending on the activated cells or the type of inflammation^{27,30,35,45} Indeed, PAR₂ agonist induces or exacerbates inflammation by a neurogenic mechanism in rat paw and mice colon by local

administration.^{31,32,46} By contrast, PAR₂ activation appears to play a protective role against inflammation in an experimental colitis model by decreasing the production of pro-inflammatory cytokines.^{26,47} This protective effect also includes a neurogenic component, because it is prevented by ablation of sensory nerves and by antagonism of CGRPs receptor.²⁶ Furthermore, PAR₂ has been reported to have a dual role in acute pancreatitis with a local protection but an aggravation of systemic complications.⁴⁸

In our experimental model, the mucosal damage, but not the leukocyte recruitment, induced by intestinal ischemia/reperfusion was significantly reduced by the intraduodenal administration of the PAR₂-selective agonist SLIGRL-NH₂ providing evidence for a protective effect of PAR₂ exogenous activation. This protective effect also seems to be partially dependent on mast cells and primary sensory afferents, because it was not observed in rats pretreated with cromolyn, a mast cell stabilizer, and chronic capsaicin, which ablates visceral afferents. As it was observed in a model of chronic colonic inflammation,²⁶ it appears from our study that activation of PAR₂ on sensory nerves can exert protective effects on intestinal mucosa.⁴⁹⁻⁵¹ This is in accord with other studies that have shown that activation of capsaicin-sensitive neurons plays an important role in protecting ischemic bowel viability.⁵²⁻⁵⁴ Because blockade of NK₁ receptor, but not CGRP receptor, reversed the protective effect on mucosal damage induced by exogenous activation of PAR₂, it is reasonable to suggest that the PAR₂-protective effect is at least in part mediated by SP released from visceral afferents. By contrast, CGRP does not appear to play an important role in this protective effect. It is likely that SP, which is overexpressed in inflammatory conditions,⁵⁵ when released by visceral afferents activates mast cells that are located in close vicinity to visceral afferents at the level of the mesentery and of the gastrointestinal mucosa,^{50,56-59} thus resulting in mast cells degranulation,

Figure 11. A and B: Real-time quantitative RT-PCR of PAR₂ mRNA expression. Shown are levels of expression of PAR₂ mRNA in upper duodenum (**A**) and distal ileum (**B**) in rats with ischemia and no reperfusion (I/R₀) treated with saline or with ischemia and 6 hours of reperfusion (I/R₆) with and without SLIGRL-NH₂ treatment. Each cDNA sample was amplified in triplicate, and all data are expressed as the mean $\pm$ SE. Real-time RT-PCR specificity was confirmed by traditional PCR as shown in the gels below each graphic. RT products from duodenum (**A**) and ileum (**B**) were used in PCR reactions with PAR₂ or β -actin primers. There was no difference among PAR₂ mRNA levels in all groups.

which accentuates the inflammatory response.⁶⁰ CGRP mediates afferent neuron mucosal protection in experimental gastritis⁵⁰ and in experimental colitis⁶¹ by modulating blood flow and vascular tone with a local action that induces nitric oxide release.⁵¹ CGRP has also been reported to exert chemotactic effect and promote mast cells recruitment.⁶² In our study, CGRP blockade does not reverse the protective effect of exogenous PAR₂ activation in intestinal ischemia, suggesting rather a pro-inflammatory role of CGRP.³¹ By contrast, in view of the increased damage score in SO rats pretreated with CGRP₈₋₃₇ compared with saline SO rats, we cannot exclude a protective role of CGRP in mild inflammatory status, as induced by laparotomy and intestinal manipulation. Because PAR₂ activation reversed the GIT delay

induced by intestinal I/R, it is reasonable to think that PAR₂ agonist could exert its protective role by modulating gastrointestinal motility. The fact that both cromolyn and capsaicin treatments inhibit both parameters (SLIGRL-NH₂-induced changes in GIT and mucosal damage) further supports this hypothesis. Because the stimulatory effect of PAR₂ activation on GIT in intestinal ischemia followed by reperfusion is not observed following blockade of either CGRP or NK₁ receptor, it is likely that visceral afferents mediate PAR₂ effect on GIT via the release of CGRP and SP.

Reperfusion of ischemic tissue results in increased production of oxidants and radicals that in turn cause mast cells activation and degranulation. These phenomena play a critical role in the cascade of events leading to I/R-induced granulocyte infiltration and mucosal barrier dysfunction.²⁸ Mast cell degranulation has been recognized as an important factor in the mediation of mucosal damage⁴⁴ and of motility alterations in the small bowel,⁶³ and it has been implicated in the pathogenesis of numerous gastrointestinal diseases, including inflammatory bowel disease, celiac disease, and food allergy.^{63,64} Moreover, mast cells activation leads to changes in intestinal motility through a neuroimmune mechanism mediated by the enteric nervous system.⁵⁷ Mast cells are the most abundant immunocytes in the gut wall, and they degranulate upon activation, releasing a multifaceted spectrum of inflammatory mediators and tryptase, a neutral serine proteinase that can activate PAR₂.⁶⁵ PAR₂ is found on enteric neurons, therefore mast cell degranulation and tryptase release may activate, in a paracrine manner, directly enteric neurons contributing to intestinal motility dysfunction and hypersecretion during intestinal inflammation.⁵⁷ However, mast cells also seem to be implicated downstream from PAR₂ activation, as suggested by the observation that pretreatment with cromolyn, a mast cell stabilizer that prevents degranulation,^{32,66} reverses the accelerating effect of PAR₂ activation on GIT in I/R animals but does not affect GIT in SO animals. This supports the concept that mast cell degranulation is one of the mechanisms involved in the PAR₂-protective effect of PAR₂ agonist on gastrointestinal motility impairment resulting from I/R.

Up-regulation of PAR₂ has been reported in response to PAR₂ activation in different experimental models of inflammation,^{27,67} which prompted our analysis of PAR₂ mRNA levels in our experimental conditions. The lack of differences in the PAR₂ mRNA levels with or without PAR₂ activation suggests that the protective effect of PAR₂ on ischemia injuries is not due to PAR₂ up-regulation, even though this cannot be ruled out. It must be pointed out that we measured PAR₂ mRNA levels 6 hours following PAR₂ stimulation, whereas in other studies, PAR₂ mRNA levels were measured 10 to 20 hours following stimulation,^{27,67} which could explain this apparent discrepancy. Indeed, PAR₂ activation results in an early down-regulation, probably due to endocytosis and degradation of the receptor, followed by an up-regulation of mRNA and protein reflecting increase of exocytosis.²⁷

In conclusion, the present findings indicate that PAR₂ activation plays a protective role on I/R-induced impair-

ment of gastrointestinal motility and mucosal damage through different mechanisms. The stimulatory effect of PAR₂ agonist on GIT in I/R is likely to be mediated by the activation of mast cells and primary visceral afferent neurons, with the involvement of both neuropeptides CGRP and SP. The inflammation caused by I/R might induce degranulation of mast cells with release of proteases that in turn activates PAR₂ directly and/or through activation of visceral afferents, thus contributing to the maintenance of regular GIT. The mechanism underlying the protective effects of PAR₂ activation on mucosal damage appears to be mediated by mast cells degranulation and sensory afferents, with a mechanism that involves at least in part SP release. PAR₂ might be an important player affecting gastrointestinal transit under stress conditions such as ischemia/reperfusion. PAR₂-related drugs could constitute useful therapeutic approaches for ischemia/reperfusion-induced impairment of gastrointestinal motility.

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References

1. Tendler DA: Acute intestinal ischemia and infarction. *Semin Gastrointest Dis* 2003, 14:66–76
2. Caplan MS, MacKendrick W: Necrotizing enterocolitis: a review of pathogenetic mechanisms and implications for prevention. *Pediatr Pathol* 1993, 13:357–369
3. Nowicki PT, Nankervis CA: The role of the circulation in the pathogenesis of necrotizing enterocolitis. *Clin Perinatol* 1994, 21:219–234
4. Massberg S, Messmer K: The nature of ischemia/reperfusion injury. *Transplant Proc* 1998, 30:4217–4223
5. Haglund U, Bergqvist D: Intestinal ischemia—the basics. *Langenbecks Arch Surg* 1999, 384:233–238
6. Collard CD, Gelman S: Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury. *Anesthesiology* 2001, 94:1133–1138
7. Huang FS, Warner BW: Necrotizing enterocolitis: a closer look. *Gastroenterology* 2001, 121:1016–1017
8. Ballabeni V, Barocelli E, Bertoni S, Impicciatore M: Alterations of intestinal motor responsiveness in a model of mild mesenteric ischemia/reperfusion in rats. *Life Sci* 2002, 71:2025–2035
9. Mallick IH, Yang W, Winslet MC, Seifalian AM: Ischemia-reperfusion injury of the intestine and protective strategies against injury. *Dig Dis Sci* 2004, 49:1359–1377
10. Todo S, Reyes J, Furukawa H, Abu-Elmagd K, Lee RG, Tzakis A, Rao AS, Starzl TE: Outcome analysis of 71 clinical intestinal transplantations. *Ann Surg* 1995, 222:270–280, discussion 280–272
11. Chan KL, Zhang XH, Fung PC, Guo WH, Tam PK: Role of nitric oxide in intestinal ischaemia-reperfusion injury studied using electron paramagnetic resonance. *Br J Surg* 1999, 86:1427–1432
12. Wiesner W, Khurana B, Ji H, Ros PR: CT of acute bowel ischemia. *Radiology* 2003, 226:635–650
13. Scudamore CL, Thornton EM, McMillan L, Newlands GF, Miller HR: Release of the mucosal mast cell granule chymase, rat mast cell protease-II, during anaphylaxis is associated with the rapid development of paracellular permeability to macromolecules in rat jejunum. *J Exp Med* 1995, 182:1871–1881
14. Bustos D, Negri G, De Paula JA, Di Carlo M, Yapur V, Facente A, De Paula A: Colonic proteinases: increased activity in patients with ulcerative colitis. *Medicina (B Aires)* 1998, 58:262–264
15. Santos J, Bayarri C, Saperas E, Nogueiras C, Antolin M, Mourelle M, Cadahia A, Malagelada JR: Characterisation of immune mediator release during the immediate response to segmental mucosal challenge in the jejunum of patients with food allergy. *Gut* 1999, 45:553–558
16. Raithe M, Winterkamp S, Pacurar A, Ulrich P, Hochberger J, Hahn EG: Release of mast cell tryptase from human colorectal mucosa in inflammatory bowel disease. *Scand J Gastroenterol* 2001, 36:174–179
17. Ossovskaya VS, Bunnett NW: Protease-activated receptors: contribution to physiology and disease. *Physiol Rev* 2004, 84:579–621
18. Thomson AB, Keelan M, Thiesen A, Clandinin MT, Ropeleski M, Wild GE: Small bowel review: diseases of the small intestine. *Dig Dis Sci* 2001, 46:2555–2566
19. Molino M, Barnathan ES, Numerof R, Clark J, Dreyer M, Cumashi A, Hoxie JA, Schechter N, Woolkalis M, Brass LF: Interactions of mast cell tryptase with thrombin receptors and PAR-2. *J Biol Chem* 1997, 272:4043–4049
20. Nystedt S, Emilsson K, Wahlestedt C, Sundelin J: Molecular cloning of a potential proteinase activated receptor. *Proc Natl Acad Sci USA* 1994, 91:9208–9212
21. Nystedt S, Emilsson K, Larsson AK, Strombeck B, Sundelin J: Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. *Eur J Biochem* 1995, 232:84–89
22. Bohm SK, Kong W, Bromme D, Smeekens SP, Anderson DC, Connolly A, Kahn M, Nelken NA, Coughlin SR, Payan DG, Bunnett NW: Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. *Biochem J* 1996, 314:1009–1016
23. Corvera CU, Dery O, McConalogue K, Gamp P, Thoma M, Al-Ani B, Caughey GH, Hollenberg MD, Bunnett NW: Thrombin and mast cell tryptase regulate guinea-pig myenteric neurons through proteinase-activated receptors-1 and -2. *J Physiol* 1999, 517:741–756
24. Cocks TM, Sozzi V, Moffatt JD, Selemidis S: Protease-activated receptors mediate apamin-sensitive relaxation of mouse and guinea pig gastrointestinal smooth muscle. *Gastroenterology* 1999, 116:586–592
25. Kawao N, Sakaguchi Y, Tagome A, Kuroda R, Nishida S, Irimajiri K, Nishikawa H, Kawai K, Hollenberg MD, Kawabata A: Protease-activated receptor-2 (PAR-2) in the rat gastric mucosa: immunolocalization and facilitation of pepsin/pepsinogen secretion. *Br J Pharmacol* 2002, 135:1292–1296
26. Fiorucci S, Mencarelli A, Palazzetti B, Distrutti E, Vergnolle N, Hollenberg MD, Wallace JL, Morelli A, Cirino G: Proteinase-activated receptor 2 is an anti-inflammatory signal for colonic lamina propria lymphocytes in a mouse model of colitis. *Proc Natl Acad Sci USA* 2001, 98:13936–13941
27. Cenac N, Coelho AM, Nguyen C, Compton S, Andrade-Gordon P, MacNaughton WK, Wallace JL, Hollenberg MD, Bunnett NW, Garcia-Villar R, Bueno L, Vergnolle N: Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. *Am J Pathol* 2002, 161:1903–1915
28. Kanwar S, Kubes P: Mast cells contribute to ischemia-reperfusion-induced granulocyte infiltration and intestinal dysfunction. *Am J Physiol* 1994, 267:G316–G321
29. Calcina F, Barocelli E, Bertoni S, Furukawa O, Kaunitz J, Impicciatore M, Sternini C: Effect of N-methyl-D-aspartate (NMDA) receptor blockade on neuronal plasticity and gastrointestinal transit delay induced by ischemia/reperfusion in rats. *Neuroscience* 2005, 134:39–49
30. Cenac N, Garcia-Villar R, Ferrier L, Larauche M, Vergnolle N, Bunnett NW, Coelho AM, Fioramonti J, Bueno L: Proteinase-activated receptor-2-induced colonic inflammation in mice: possible involvement of afferent neurons, nitric oxide, and paracellular permeability. *J Immunol* 2003, 170:4296–4300
31. Steinhoff M, Vergnolle N, Young SH, Tognetto M, Amadesi S, Ennes HS, Trevisani M, Hollenberg MD, Wallace JL, Caughey GH, Mitchell SE, Williams LM, Geppetti P, Mayer EA, Bunnett NW: Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. *Nat Med* 2000, 6:151–158
32. Vergnolle N, Hollenberg MD, Sharkey KA, Wallace JL: Characterization of the inflammatory response to proteinase-activated receptor-2 (PAR2)-activating peptides in the rat paw. *Br J Pharmacol* 1999, 127:1083–1090
33. Kato K, Martinez V, St Pierre S, Tache Y: CGRP antagonists enhance gastric acid secretion in 2-h pylorus-ligated rats. *Peptides* 1995, 16:1257–1262

34. Kato K, Yang H, Tache Y: Role of peripheral capsaicin-sensitive neurons and CGRP in central vagally mediated gastroprotective effect of TRH. *Am J Physiol* 1994, 266:R1610–R1614
35. Nguyen C, Coelho AM, Grady E, Compton SJ, Wallace JL, Hollenberg MD, Cenac N, Garcia-Villar R, Bueno L, Steinhoff M, Bunnett NW, Vergnolle N: Colitis induced by proteinase-activated receptor-2 agonists is mediated by a neurogenic mechanism. *Can J Physiol Pharmacol* 2003, 81:920–927
36. Garret C, Caruette A, Fardin V, Moussaoui S, Peyronel JF, Blanchard JC, Laduron PM: Pharmacological properties of a potent and selective nonpeptide substance P antagonist. *Proc Natl Acad Sci USA* 1991, 88:10208–10212
37. Izzo AA, Pinto L, Borrelli F, Capasso R, Mascolo N, Capasso F: Central and peripheral cannabinoid modulation of gastrointestinal transit in physiological states or during the diarrhoea induced by croton oil. *Br J Pharmacol* 2000, 129:1627–1632
38. McCafferty DM, Sihota E, Muscara M, Wallace JL, Sharkey KA, Kubes P: Spontaneously developing chronic colitis in IL-10/iNOS double-deficient mice. *Am J Physiol* 2000, 279:G90–G99
39. Vergnolle N, Cellars L, Mencarelli A, Rizzo G, Swaminathan S, Beck P, Steinhoff M, Andrade-Gordon P, Bunnett NW, Hollenberg MD, Wallace JL, Cirino G, Fiorucci S: A role for proteinase-activated receptor-1 in inflammatory bowel diseases. *J Clin Invest* 2004, 114:1444–1456
40. Anselmi L, Lakhter A, Hirano AA, Tonini M, Sternini C: Expression of galanin receptor messenger RNAs in different regions of the rat gastrointestinal tract. *Peptides* 2005, 26:815–819
41. Hebra A, Brown MF, McGeehin K, Broussard D, Ross AJ 3rd: The effects of ischemia and reperfusion on intestinal motility. *J Pediatr Surg* 1993, 28:362–365; discussion 365–366
42. Udassin R, Eimerl D, Schiffman J, Haskel Y: Postischemic intestinal motility in rat is inversely correlated to length of ischemia. *An in vivo animal model*. *Dig Dis Sci* 1995, 40:1035–1038
43. Lindstrom LM, Ekblad E: Structural and neuronal changes in rat ileum after ischemia with reperfusion. *Dig Dis Sci* 2004, 49:1212–1222
44. Boros M, Kaszaki J, Ordogh B, Nagy S: Mast cell degranulation prior to ischemia decreases ischemia-reperfusion injury in the canine small intestine. *Inflamm Res* 1999, 48:193–198
45. Vergnolle N: Protease-activated receptors and inflammatory hyperalgesia. *Mem Inst Oswaldo Cruz* 2005, 100(Suppl 1):173–176
46. Lindner JR, Kahn ML, Coughlin SR, Sambrano GR, Schauble E, Bernstein D, Foy D, Hafezi-Moghadam A, Ley K: Delayed onset of inflammation in protease-activated receptor-2-deficient mice. *J Immunol* 2000, 165:6504–6510
47. Fiorucci S, Distrutti E: Role of PAR2 in pain and inflammation. *Trends Pharmacol Sci* 2002, 23:153–155
48. Namkung W, Han W, Luo X, Muallem S, Cho KH, Kim KH, Lee MG: Protease-activated receptor 2 exerts local protection and mediates some systemic complications in acute pancreatitis. *Gastroenterology* 2004, 126:1844–1859
49. Reinshagen M, Flamig G, Ernst S, Geerling I, Wong H, Walsh JH, Eysselein VE, Adler G: Calcitonin gene-related peptide mediates the protective effect of sensory nerves in a model of colonic injury. *J Pharmacol Exp Ther* 1998, 286:657–661
50. Holzer P, Lippe IT: Role of calcitonin gene-related peptide in gastrointestinal blood flow. *Ann NY Acad Sci* 1992, 657:228–239
51. Lambrecht N, Burchert M, Respondek M, Muller KM, Peskar BM: Role of calcitonin gene-related peptide and nitric oxide in the gastroprotective effect of capsaicin in the rat. *Gastroenterology* 1993, 104:1371–1380
52. Pawlik WW, Thor P, Sendur R, Biernat J, Kozioł R, Wasowicz P: Myoelectric bowel activity in ischemia/reperfusion damage. Role of sensory neurons. *J Physiol Pharmacol* 1998, 49:543–551
53. Brzozowska I, Targosz A, Sliwowski Z, Kwiecien S, Drozdowicz D, Pajdo R, Konturek PC, Brzozowski T, Pawlik M, Konturek SJ, Pawlik WW, Hahn EG: Healing of chronic gastric ulcers in diabetic rats treated with native aspirin, nitric oxide (NO)-derivative of aspirin and cyclooxygenase (COX)-2 inhibitor. *J Physiol Pharmacol* 2004, 55:773–790
54. Dun Y, Hao YB, Wu YX, Zhang Y, Zhao RR: Protective effects of nitroglycerin-induced preconditioning mediated by calcitonin gene-related peptide in rat small intestine. *Eur J Pharmacol* 2001, 430:317–324
55. Pidsudko Z, Wasowicz K, Sienkiewicz W, Kalczyk J, Czaja K, Lakomy M: The influence of inflammation on the expression of neuropeptides in the ileum-projecting primary sensory neurones in the pig. *Folia Morphol (Warsz)* 2003, 62:235–237
56. Crivellato E, Damiani D, Mallardi F, Travan L: Suggestive evidence for a microanatomical relationship between mast cells and nerve fibres containing substance P, calcitonin gene related peptide, vasoactive intestinal polypeptide, and somatostatin in the rat mesentery. *Acta Anat (Basel)* 1991, 141:127–131
57. Sharkey KA, Mawe GM: Neuroimmune and epithelial interactions in intestinal inflammation. *Curr Opin Pharmacol* 2002, 2:669–677
58. Stead RH, Tomioka M, Quinonez G, Simon GT, Felten SY, Bienenstock J: Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. *Proc Natl Acad Sci USA* 1987, 84:2975–2979
59. Skofitsch G, Savitt JM, Jacobowitz DM: Suggestive evidence for a functional unit between mast cells and substance P fibers in the rat diaphragm and mesentery. *Histochemistry* 1985, 82:5–8
60. Spiller RC: Role of nerves in enteric infection. *Gut* 2002, 51:759–762
61. McCafferty DM, Wallace JL, Sharkey KA: Effects of chemical sympathectomy and sensory nerve ablation on experimental colitis in the rat. *Am J Physiol* 1997, 272:G272–G280
62. De Jonge F, Van Nassauw L, Adriaensen D, Van Meir F, Miller HR, Van Marck E, Timmermans JP: Effect of intestinal inflammation on capsaicin-sensitive afferents in the ileum of *Schistosoma mansoni*-infected mice. *Histochem Cell Biol* 2003, 119:477–484
63. Stenton GR, Vliagoftis H, Befus AD: Role of intestinal mast cells in modulating gastrointestinal pathophysiology. *Ann Allergy Asthma Immunol* 1998, 81:1–11, quiz 12–15
64. Crowe SE, Perdue MH: Gastrointestinal food hypersensitivity: basic mechanisms of pathophysiology. *Gastroenterology* 1992, 103:1075–1095
65. Payne V, Kam PC: Mast cell tryptase: a review of its physiology and clinical significance. *Anaesthesia* 2004, 59:695–703
66. Mazurek N, Berger G, Pecht I: A binding site on mast cells and basophils for the anti-allergic drug cromolyn. *Nature* 1980, 286:722–723
67. Olejar T, Matej R, Zadinova M, Pouckova P: Expression of proteinase-activated receptor 2 during taurocholate-induced acute pancreatic lesion development in Wistar rats. *Int J Gastrointest Cancer* 2001, 30:113–121