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Proteinase-Activated Receptor-2-Induced Colonic Inflammation in Mice: Possible Involvement of Afferent Neurons, Nitric Oxide, and Paracellular Permeability

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Activation of colonic proteinase-activated receptor-2 (PAR-2) provokes colonic inflammation and increases mucosal permeability in mice. The mechanism of inflammation is under debate and could be neurogenic and/or the consequence of tight-junction opening with passage of exogenous pathogens into the lamina propria. The present study aimed to further characterize the inflammatory effect of PAR-2 activation by investigating: 1) the role of NO, 2) the role of afferent neurons, and 3) a possible cause and effect relationship between colonic paracellular permeability changes and mucosal inflammation. Thus, intracolonic infusion to mice of the PAR-2-activating peptide, SLIGRL, increased both myeloperoxidase (MPO) activity and damage scores indicating colonic inflammation, and enhanced colonic permeability to 51Cr-EDTA from 2 to 4 h after its infusion. NO synthase inhibitors, L-NAME and aminoguanidine, as well as the neurotoxin capsaicin and NK1, calcitonin gene-related peptide (CGRP) receptor antagonists, SR140333 and CGRP8-37, prevented SLIGRL-induced MPO and damage score increases and permeability. In contrast, although the tight-junction blocker, 2,4,6-triaminopyrimidine, and the myosin L chain kinase inhibitor, ML-7, prevented SLIGRL-induced increase in permeability, they did not prevent MPO and damage score increases. Taken together our data show that both NO and capsaicin-sensitive afferent neurons are involved in PAR-2-mediated colonic inflammation and paracellular permeability increase. Nevertheless, the inflammation process is not a consequence of increased permeability which results at least in part from the activation of myosin L chain kinase.


Proteinase-activated receptors (PARs)3 belong to a family of seven transmembrane domain G-protein-coupled recep-
tors that are activated by cleavage of their N-terminal do-
main by a proteolytic enzyme (1). The unmasked new N-terminal sequence acts as a tethered ligand that binds and activates the receptor itself. PAR-2 is activated by trypsin and mast cell trypstatine, but synthetic peptides, so-called PAR-activating peptides, (NH2-SLIGRL for PAR-2, hereafter referred to as SLIGRL) cor-
responding to the tethered ligand sequence, are also able to selectively activate PARs (2). PAR-2 is expressed throughout the gastroenteral tract on several cell types, including enterocytes, mast cells, smooth muscle cells, myenteric neurons, and endothelial cells (3). Indeed, at concentrations physiologically present in the intestinal lumen, trypsin and trypsin-like molecules activate PAR-2 at the apical membrane of enterocytes and stimulate the generation of inositol-1,4,5-triphosphate, arachidonic acid release, and secretion of eicosanoids, which may in turn influence the intestinal function (3). Moreover, PAR-2 has been shown involved in pathophysiological processes. Thus, in vivo PAR-2-AP injection into the paw of rats produced an inflammatory reaction by a neurogenic mechanism involving afferent capsaicin-sensitive sensory neurons and both calcitonin gene-related peptide (CGRP) and substance P (SP) release (4). SLIGRL infused directly into the colonic lumen of rats activates nociceptive neurons and causes delayed rectal hyperalgesia which involves the peripheral activation of tachykinin NK1 receptors (5). We have previously shown that intracolonic infusion of SLIGRL, trypsin, or trypstatine to mice led to colonic inflammation characterized by granulocyte infiltration revealed by elevated levels of MPO activity, tissue damage, and increased expression of proinflammatory cytokines (6). Moreover, the cytokine profile observed after PAR-2-AP-induced inflammation revealed a Th1-like type of inflammation, in which TNF-α, IL-1β, and IFN-γ mRNA levels are elevated, while IL-4 and IL-10 levels remain stable. This intestinal inflammation was accompanied by changes in paracellular permeability marked by an increased passage of labeled 51Cr-EDTA and the presence of bacteria translocated to peritoneal organs (6). The mechanism(s) triggered by PAR-2 activation are not clearly understood. Either, this PAR-2-mediated increase of colonic paracellular permeability might constitute an initial event, which then allows penetration of pathogens to cause further inflammation or intestinal inflammation can enhance epithelial barrier breakdown like in ulcerative colitis and Crohn’s disease (7, 8). Inducible NO synthase (iNOS) activity was dramatically increased in colonic biopsies from inflammatory

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3 Abbreviations used in this paper: PAR, proteinase-activated receptor; MPO, myeloperoxidase; MLCK, myosin L chain kinase; iNOS, inducible NO synthase; EGTA, ethylene-glycol-bis(o-aminomethyl)-N,N,N’,N’-tetraacetic acid; eNOS, constitutive NOS.

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bowel disease (IBD) patients. A correlation between this activity and the severity of bowel inflammation was found for ulcerative colitis (9). In the intestine, NO can be produced by epithelial cells (10) or nonadrenergic noncholinergic nerves (11) and the effects of NO on intestinal epithelial integrity (12) and permeability (13) have been reported. In fact, inhibition of NO production leads to a reversible circulating leukocyte-independent increase in epithelial permeability. Moreover, down-regulation of junctional proteins and their respective mRNA is observed in actively inflamed colon of patients with IBD, while in tissues from inactive IBD patients, only few junctional molecules are affected, and expression of tight junction-associated proteins appears almost unchanged (5).

Consequently, the aims of this work were 3-fold: first, to further characterize the mechanisms of PAR-2-induced colonic inflammation by investigating the involvement of capsaicin sensitiveafferent neurons and NO; second, to address the issue of a possible cause and effect relationship between the enhancement of paracellular permeability and mucosal inflammation mediated by PAR-2 activation by using a chemical blocker of tight-junctions; and third, to investigate the role of epithelial cell cytoskeleton protein contractility in the PAR-2-mediated increase of paracellular permeability, using a myosin L chain kinase (MLCK) inhibitor.

Materials and Methods

Animals

Male Swiss 3T3 mice were obtained respectively from Harlan (Gannat, France) and Iffa-Credo (L’Arbresle, France). Mice were housed in polycarbonate cages in a light (12 h/12 h cycle)- and temperature-controlled room (20–22°C) and were fed standard pellets asine d’alimentation rationnelle (UAR) A03 (Epinay-sur-Orge, France). Water was provided ad libitum. Four groups of eight mice received the neurotoxin capsaicin (25 mg/kg in 100 μl twice daily for 2 days) or its vehicle (saline) and were treated 30 min later by SLIGRL (100 μg/mouse) or saline. Two groups of 10 mice received the neurotoxin capsaicin (25 mg/kg in 100 μl) twice daily for 2 days) or its vehicle (saline), and the rest of the body were placed in separate counting tubes in a gamma-counter (Packard Cobra II; Packard Bioscience, Rungis, France). The permeability was expressed as the ratio between body and total (body plus colon) radioactivities.

NOS activity assay

Tissue NOS activities were determined by measuring the rate of conversion of l-arginine to l-citrulline using the method of Bush (19) modified by Anto et al. (20). Briefly, tissue samples were homogenized on ice in buffer (pH 7.4) containing Tris-HCl (50 mM), DTT (1 mM), phenylmethylsulfonyl fluoride (1 mM), EDTA (0.1 mM), and two protease inhibitors: leupeptin (23.4 μM) and pepstatin (14.6 μM). After centrifugation (13,500 g, 30 min, 4°C), 100 μl of supernatant was added to the reaction mixture containing 50 mM Tris-HCl (pH 7.4), 1.58 μM L-arginine, DTT (1 mM), valine (50 mM), 200 μM NADPH as a coenzyme, and 10 μM flavo mononucleotide and 10 μM flavomamine diucleotide as prosthetic groups of NOS. Determination of total NOS activity (i.e., NOS-specific activity and nonspecific activity) was performed by adding 2 mM CaCl2 to the buffer. NOS activity was determined in the presence of a calcium chelator, ethyleneglycol-bis(α-aminoethyl)-N,N,N’,N’-tetraacetic acid (EGTA, 1 mM). Nonspecific activity was determined in the presence of 1 mM EGTA and 20 mM L-NAME, a nonspecific NOS inhibitor. After 30 min of incubation at 37°C, the enzymatic reaction was stopped by adding cold HEPES buffer (pH 5.5) containing 1 mM EGTA and 1 mM EDTA, l-citrulline formed in the medium was separated by applying the samples to columns containing pre-equilibrated Dowex AG50W-X8 (Sigma-Aldrich, St. Quentin Fallavier, France), eluting them with water, and measuring the amount of radioactivity with a liquid scintillation beta counter (Kontron Instruments, St Quentin en Yvelines, France). Total NOS specific (i.e., NOS + constitutive NOS (cNOS)) activity was determined by the difference between the l-citrulline generated in samples containing 2 mM CaCl2 and samples containing 1 mM EGTA and 20 mM l-NAME, the cNOS activity was determined from the difference between total specific NOS activity and iNOS activity in samples containing 1 mM EGTA. Enzyme activity was expressed as picomoles of citrulline formed per milligram of protein per hour.

Chemicals

Peptides (SLIGRL-NH2, and LRGILS-NH2) prepared by solid phase synthesis were obtained from NeoSystem (Strasbourg, France). The composition and purity of peptides were confirmed by HPLC analysis. SLIGRL was dissolved in 10% ethanol, 10% Tween 80, and 80% saline. SR140333 was graciously given by Dr. X. Edmond-Alt (Sano-Synthelabo, Montpellier, France) and was dissolved in 50% saline and 50% DMSO. CGRP43-52, capsaicin, aminoguanidine, t-NAME, ML-7, and TAP were obtained from Sigma-Aldrich (St. Quentin Fallavier, France). Aminoguanidine and t-NAME were dissolved in saline. Capsaicin was dissolved in 20% ethanol, 10% Tween 80, and 70% saline. ML-7 was dissolved in 2% ethanol and 98% saline. TAP was dissolved in Tris-HCl (0.1 M) buffer solution.

Statistical analysis

Data are presented as means ± SEM, and statistical significance was assessed by ANOVA followed by Tukey’s multiple comparison test or by Student’s t test where appropriate. Values of p < 0.05 were considered significant.

Results

Values obtained with the different treatments were normalized to SLIGRL values considered as 100% unless otherwise indicated.
Effects of NK1 and CGRP receptor antagonists, and sensory denervation on PAR-2-AP-mediated changes in colonic paracellular permeability and inflammation

Antagonism of NK1 receptor by SR140333 or antagonism of CGRP receptor by CGRP$_{8-37}$ or depletion of afferent fibers by s.c. administration of capsaicin (25 mg/kg) twice daily for 2 days, 1 wk before peptide administration, prevented SLIGRL-induced increase of MPO activity (respectively, 12.1 ± 3.8, 15.2 ± 2.5, and 3.3 ± 0.6%; p < 0.01) (Fig. 1A) and damage score (11.8 ± 2.5, 14.2 ± 4.9, and 6.1 ± 1.6%; p < 0.01) (Fig. 1B), indicating inhibition of the inflammatory process. Moreover, PAR-2-AP-induced increase in paracellular permeability was significantly reduced in SR140333, CGRP$_{8-37}$, and capsaicin-treated mice (35.4 ± 3.1, 42.6 ± 2.9, and 37.5 ± 3.1%; p < 0.05) (Fig. 1C). SR140333, CGRP$_{8-37}$, or capsaicin have no per-se effect on MPO, damage scores, and colonic permeability.

Effects of PAR-2-AP on colonic NOS activity

Intracolonic administration of SLIGRL (100 μg/mouse) but not the reverse peptide LRGILS (100 μg/mouse) caused a significant increase of iNOS activity (60.1 ± 10.9 vs 12.2 ± 0.8 fmol/mg/h) and a significant decrease of eNOS activity (9.2 ± 0.5 vs 20.1 ± 9.1 fmol/mg/h) (Table I).

Effects of NOS inhibitors on PAR-2-AP-mediated changes in colonic paracellular permeability and inflammation

Intraperitoneal injection of the iNOS activity inhibitor, aminoguanidine (50 mg/kg), or the nonisoform-specific NOS inhibitor, L-NAME (20 mg/kg), 30 min before SLIGRL administration reduced the PAR-2-AP-induced inflammation as it prevented the increase in MPO activity (14.26 ± 3.4 and 18.9 ± 4.2%, respectively; p < 0.05) (Fig. 1A) and reduced scores (34.3 ± 1.8 and 42.1 ± 4.1%; p < 0.05) (Fig. 1B). Moreover, PAR-2-AP-induced increase of paracellular permeability was significantly reduced by aminoguanidine and L-NAME (50.9 ± 4.3 and 34.1 ± 6.4%; p < 0.05) (Fig. 1C). L-NAME or aminoguanidine did not affect basal MPO, scores, and colonic permeability by themselves.

Effects of tight junction blockade on PAR-2-AP-mediated changes in colonic paracellular permeability and inflammation

The tight junction blocker, TAP, infused intracolonically between 0 and 4 h after peptide administration abolished SLIGRL-induced increase in paracellular permeability to $^{51}$Cr-EDTA (20.6 ± 7.4%; p < 0.01) (Fig. 1C). However, TAP did not prevent the inflammation process as indicated by similar values of both MPO (87.6 ± 17.1%) (Fig. 1A) and damage scores (96.3 ± 18.4%) compared with SLIGRL alone (Fig. 1B). TAP alone did not affect basal MPO, scores, and colonic permeability.

Discussion

The present study confirms our previous observation that intracolonic administration of the PAR-2-activating peptide, SLIGRL, results in colonic inflammation and increase in paracellular permeability (6). Our present results bring novel data on the mechanisms involved in both effects.

First, colonic inflammation mediated by PAR-2 activation involves capsaicin-sensitive afferent neurons and neuromediators such as SP and CGRP. Indeed, appropriate pretreatment with the neurotoxin capsaicin inhibits the different hallmarks of inflammation such as the increase of tissue MPO activity, a marker of neutrophil infiltration, and the enhancement of microscopic damage scores induced by intracolonic SLIGRL. In addition, PAR-2-AP-induced loosening of the mucosal epithelial barrier is also reduced by capsaicin treatment, confirming that both effects of PAR-2 activation on colonic mucosa are neurally mediated through afferent neurons.

Second, a marked reduction of SLIGRL-induced colonic inflammation and permeability was observed after aminoguanidine treatment, suggesting a major role of iNOS, in agreement with the increase in iNOS activity and a decrease in eNOS activity observed in our study after intracolonic administration of SLIGRL.

Third, we also provided evidence in this study that SLIGRL affects epithelial cell permeability by provoking a contraction of

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**Table 1. Effect of intracolonic administration of SLIGRL on NOS activity**

<table>
<thead>
<tr>
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<th>Control</th>
<th>SLIGRL</th>
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</thead>
<tbody>
<tr>
<td>cNOS (fmol/mg/h)</td>
<td>20.1 ± 9.1</td>
<td>9.2 ± 0.5*</td>
</tr>
<tr>
<td>iNOS (fmol/mg/h)</td>
<td>12.2 ± 0.8</td>
<td>60.1 ± 10.9*</td>
</tr>
</tbody>
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*a Expressed in femtomoles per milligrams per hour of l-arginine converted into NO. Values are means ± SEM (n = 10).

* p < 0.05 compared to corresponding control.
the cytoskeleton. To investigate a possible cause and effect relationship between the enhancement of paracellular permeability and mucosal inflammation mediated by PAR-2 activation, we used a chemical tight-junction blocker (TAP). While SLIGRL-induced increase in paracellular permeability is prevented by TAP, colonic inflammation is unaffected by this compound, leading to the conclusion that the inflammatory reaction of the lamina propria did not result from an increased paracellular permeability and subsequent penetration of potential pathogens from the lumen. We have further characterized PAR-2-mediated modulation of permeability by using a specific MLCK inhibitor, ML-7. Similarly, PAR-2-AP-induced increase of paracellular permeability is blocked by ML-7 but the heralding signs of inflammation are still present. Taken together, these observations suggest that SLIGRL-induced inflammation does not result from altered tight-junction permeability. These data also indicated that activation of MLCK is probably involved in PAR-2-induced colonic permeability increase.

Under physiological conditions, NO is essentially produced by eNOS and plays an anti-inflammatory role, maintaining mucosal integrity by tonic secretion of low quantities of NO (21). In contrast, lack of eNOS activity or excess of iNOS activity may lead to gut inflammation (22). In a rat model of acute colitis (23) and in biopsies from patients with active ulcerative colitis or Crohn’s disease, colonic iNOS activity was found to be significantly increased, whereas cNOS activity was decreased (24). Our data show that colonic PAR-2 activation increased iNOS activity and decrease cNOS activity, and the fact that a similar protection from PAR-2-induced inflammation and permeability changes was achieved by treatment of mice with aminoxyguanidine, supports a major involvement of increased iNOS in the proinflammatory influence of local activation of PAR-2. Our findings are consistent with several studies showing attenuated colonic injury in different models of intestinal inflammation treated with iNOS inhibitors (20).

Whereas both sympathetic and parasympathetic afferent nerve activity has a modulatory function in peripheral inflammatory processes, the afferent neurons have herein a major role in SLIGRL-induced inflammation. In a basal state, the stimulation of afferent neurons produces symptoms similar to those observed during inflammation: vasodilatation, plasma extravasation, platelet accumulation and aggregation, and mast cell activation. Capsaicin-sensitive fibers contain notably tachykinins, like SP and neurokinin A, and CGRP. CGRP seems to be mainly responsible for the increase in blood flow, whereas SP and neurokinin A account for vascular leakage. A high proportion of visceral afferent neurons contain SP and its NK-1 receptor, which are involved in the mechanism of visceral pain. A study demonstrated that peripheral NK-1 receptors are implicated in PAR-2-AP-induced delayed rectal hyperalgesia and hypothesized that SLIGRL activates directly a local release of SP from primary spinal afferent neurons through an axonal reflex (5). SP can trigger many events associated with neurogenic inflammation and the release of algogenic mediators from recruited immune cells. Our present study confirms these hypotheses for visceral inflammation, previous observation made on rat paw edema model in which a diminution of PAR-2-mediated edema also occurs after destroying capsaicin-sensitive afferent fibers by chronic treatment with high doses of capsaicin. Nevertheless, in this model, the mediators released by sensory nerves also protect against the inflammatory effects of the PAR-2 agonist, because ablation of sensory nerves enhanced PAR-2-induced infiltration of neutrophils into the paw (4).

In contrast to basal state in which activation of capsaicin afferent fiber promotes a neurogenic inflammation, we have previously shown in an experimental model of colitis in rats that capsaicin deafferentation and more specifically vagal deafferentation increased the inflammatory response, indicating that vagal afferences exert a protective role in this model (25). PAR-2 activation as acute stimulation of afferences by capsaicin, reduces the inflammatory response in a chronic colitis model in rat (26). It was also demonstrated that administration of CGRP8-37 or ablation of sensory nerves with capsaicin reverses the protection exerted by PAR-2 in this model confirming that PAR-2 protects from inflammation through activation of sensory nerves (26). Finally, our data do not contrast the literature supporting that in basal state, stimulation of efferent nerve occurs a neurogenic inflammation and during the course of inflammation has protective effects (26).

Treatment by capsaicin also reduces the increase in paracellular permeability in response to the PAR-2 agonist, suggesting that capsaicin-sensitive afferent neurons are involved in such effect. However, capsaicin does not completely suppress the increase in paracellular permeability triggered by PAR-2 activation suggesting a possible nonneurogenic component in this effect of PAR-2 on permeability.

Interestingly, blockade of SLIGRL-induced paracellular permeability by TAP did not affect the inflammatory response indicating that SLIGRL induced inflammation is not a consequence of epithelial barrier breakdown. To further characterize the pathway by which SLIGRL affects tight-junctions, we used ML-7, a MLCK inhibitor. ML-7 prevented the effect of SLIGRL on paracellular permeability suggesting that PAR-2 activates MLCK to phosphor-ylate myosin L chain, leading to the contraction of epithelial cell cytoskeleton responsible of tight-junction opening (27). PAR-2 may activate directly MLCK in enterocytes by increasing intracellular Ca2+ (3). However SLIGRL-induced MLCK activation and/or myosin L chain phosphorylation may be modulated by other intracellular pathways involving mitogen-activated protein kinase (28) or Rho kinase (29). Indeed, by a mechanism that requires receptor association into a multiprotein complex, SLIGRL-NH2 favors the internalization of PAR-2 by β-arrestin and activation of extracellular signal-regulated kinase 1/2 in bRIEC cells transfected with human PAR-2 which can enhance MLCK activity (30). This hypothesis is supported by the internalization of the receptor in enterocytes after exposure to SLIGRL-NH2 (6). Finally, our data clearly show that distinct pathways exist for SLIGRL-induced alterations in inflammation and permeability. However, we cannot exclude that increased permeability is the consequence of a primary neurogenic inflammation. In a previous study performed in rats, we have shown that PAR-2 activation initiates a delayed rectal hyperalgesia associated with increased colonic tight junction permeability to 51Cr-EDTA but without detectable inflammation (5). This result suggests that increased paracellular permeability is of paramount importance to trigger visceral pain independently of any inflammatory reaction. We have also previously reported that activation of colonic PAR-2 increased proinflammatory cytokines and induced a Th-1 profile characterized by elevated levels of IFN-γ mRNA (6). In vitro, it has been shown that IFN-γ disrupts the epithelial barrier function of T84 cells by decreasing the levels of zonaula occludens-1, perturbing the actin cytoskeleton in the tight junction area, and causing the mis-localization of zonaula occludens-2 and occludin (31). In addition, TNF-α has been reported to promote the disturbing effects of IFN-γ on the epithelial barrier (32).

In summary, both NO and capsaicin-sensitive afferent neurons are involved in PAR-2-mediated colonic inflammation and increase in paracellular permeability. Nevertheless, the inflammation process is independent of the increase of paracellular permeability which involves MLCK modulation.
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