PAR-2-mediated control of barrier function and motility differs between early and late phases of postinfectious gut dysfunction in the rat

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Proteinase-activated receptor-2 (PAR-2) and mast cell (MC) mediators contribute to inflammatory and functional gastrointestinal disorders. We aimed to characterize jejunal PAR-2-mediated responses and the potential MC involvement in the early and late phases of a rat model of postinfectious gut dysfunction. Jejunal tissues of control and Trichinella spiralis-infected (14 and 30 days postinfection) rats, treated or not with the MC stabilizer, ketotifen, were used. Histopathology and immunostaining were used to characterize inflammation, PAR-2 expression, and mucosal and connective tissue MCs. Epithelial barrier function (hydro-electrolytic transport and permeability) and motility were assessed in vitro in basal conditions and after PAR-2 activation. Intestinal inflammation on day 14 postinfection (early phase) was significantly resolved by day 30 (late phase) although MC counts and epithelial permeability remained increased. PAR-2-mediated ion transport (Ussing chambers, in vitro) and epithelial surface PAR-2 expression were reduced in the early phase, with a trend toward normalization during the late phase. In control conditions, PAR-2 activation (organ bath) induced biphasic motor responses (relaxation followed by excitation). At 14 days postinfection, spontaneous contractility and PAR-2-mediated relaxations were enhanced; motor responses were normalized on day 30. Postinfectious changes in PAR-2 functions were not affected by ketotifen treatment. We concluded that, in the rat model of Trichinella spiralis infection, alterations of intestinal PAR-2 function and expression depend on the inflammatory phase considered. A lack of a ketotifen effect suggests alterations observed in several gastrointestinal disorders, in particular spontaneous and PAR-2-mediated intestinal hydro-electrolytic transport, paracellular permeability, and contractility were assessed in vitro. In parallel, PAR-2 immunostaining was performed to elucidate whether the functional changes observed were related to alterations in the receptor expression. In addition, we studied the abundance of intestinal mucosal and connective tissue MC populations, which express proteinases able to modulate intestinal functions and to activate PAR-2 (1, 28, 29). Finally, to assess a potential role of MC-derived proteinases directly in postinfectious gut dysfunction, the influence of MC stabilization during the course of infection on epithelial barrier function (EBF) and contractility was assessed.

MATERIALS AND METHODS

Animals

Adult male OFA Sprague-Dawley rats (7–8 wk old, 250–275 g; Charles River Laboratories, Lyon, France) were used. Rats were housed under conventional conditions in a light (12-h:12-h light/dark cycle) and temperature-controlled (20–22°C) room, with access to tap

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It is well known that proteinases act as signaling molecules by cleaving and activating proteinase-activated receptor (PAR) family members (PAR-1, -2, -3, and -4). In particular, PAR-2 can be activated by endogenous and exogenous proteinases, including mast cell (MC)-derived tryptase and bacterial- and parasite-derived proteinases. Within the gut, PAR-2 activation has been shown to modulate inflammation, nociception, permeability, motility, and ion transport (1, 13, 33). Accordingly, PAR-2 and its activating proteinases have been suggested to participate in intestinal diseases characterized by sensory and secretomotor alterations, such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). This idea was further reinforced when changes in the levels of expression of PAR-2 and a role for PAR-2 were reported in patients with IBD and IBS (5, 11, 16, 18, 38, 40). However, the mechanisms underlying these expression changes and their functional significance are as yet not completely understood.

MCs have been suggested as effector cells in the functional alterations observed in several gastrointestinal disorders, including IBS or intestinal inflammation (30). Because MCs from humans and other species release a variety of proteinases upon activation and degranulation, a link between MCs and PAR-2 activation has been suggested. Indeed, some data indicate that MC-derived proteinases can act as local activators of PAR-2, leading to PAR-2-mediated functional responses (22, 29, 33, 46). In contrast, other work suggests that, because of NH2-terminal PAR-2 receptor glycosylation, MC-derived proteinases, like tryptase, are restricted in their ability to activate PAR-2 (6).

The aim of the present work was to further investigate the interplay between PAR-2 and MCs during intestinal inflammation and dysfunction. For this purpose, we used Trichinella spiralis (T. spiralis)-infected rats, a validated model of MC-associated enteritis and gut dysfunction that is regarded as a valid model of IBS (7, 10, 35, 43). We studied how intestinal function was affected under basal conditions and after PAR-2 activation in the early and the late postinfectious phases. In particular, spontaneous and PAR-2-mediated intestinal hydro-electrolytic transport, paracellular permeability, and contractility were assessed in vitro. In parallel, PAR-2 immunostaining was performed to elucidate whether the functional changes observed were related to alterations in the receptor expression. In addition, we studied the abundance of intestinal mucosal and connective tissue MC populations, which express proteinases able to modulate intestinal functions and to activate PAR-2 (1, 28, 29). Finally, to assess a potential role of MC-derived proteinases directly in postinfectious gut dysfunction, the influence of MC stabilization during the course of infection on epithelial barrier function (EBF) and contractility was assessed.
water and laboratory rat chow ad libitum. Animals were kept in groups of two to three per cage, except otherwise stated. All experimental procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocols 5352 and 5564).

**T. spiralis Infection**

Muscle-stage larvae of *T. spiralis* were obtained from infected CD1 mice as previously described (4, 35, 43). Rats were infected at 8–9 wk of age by administration of 7,500 larvae per mouse. Age-matched rats dosed orally with 1 ml of saline were used as controls. During this time, animals were regularly monitored for clinical signs and body weight changes. Normal course of the infection was confirmed by a significant decrease of body weight after *T. spiralis* infection compared with controls, with a peak reduction on day 9 and a subsequent linear increase over time, as previously described by us (10, 43).

**Ketotifen Treatment**

For the stabilization of MCs, ketotifen was added to the drinking water. The compound was dissolved in water at an initial concentration of 0.1 mg/ml, allowing an estimated dosage of 10 mg/kg per day (taking a mean water consumption of 30 ml/day). During the treatment period, animals were housed individually, and the amount of water drunk by each rat was monitored daily and the concentration of ketotifen adjusted to ensure the desired dosage. Ketotifen solutions were freshly prepared every 2 days. Similar treatment protocols have been used before, both clinically and preclinically, showing effects consistent with a ketotifen-mediated modulation of MC degranulation (14, 19, 35, 42, 45).

**Tissue Sampling**

At the time of the experiments (either at day 14 or day 30 postinfection), animals were euthanized by decapitation, a laparotomy was performed, and jejunal samples (beginning 10 cm distal to the ligament of Treitz) were obtained. For functional studies (motility and barrier function), jejunal segments were immediately flushed and placed in ice-cold oxygenated Krebs buffer containing glucose. At the same time, jejunal samples for histological and immunostaining studies were obtained and fixed in 4% paraformaldehyde in phosphate buffer for 24 h. Thereafter, fixed samples were processed routinely for paraffin embedding, and 5-μm sections were obtained for hematoxylin and eosin (H&E) staining, rat MC proteinase 2 (rMCP-2) chymase or rMCP-6 tryptase immunohistochemistry, or PAR-2 immunofluorescence.

**Histopathological Studies**

H&E-stained slides were evaluated in a blinded fashion by two independent investigators. A histopathological score (ranging from 0, normal, to 18, maximal alterations) was assigned to each animal. Specifically, parameters scored included the following: epithelial structure (0: normal; 1: mild alterations of the villi; 2: local villi destruction and/or fusion and/or local destruction of crypts; 3: generalized villi destruction and/or fusion and/or generalized destruction of crypts), presence of edema (0: normal; 1: mild local edema in submucosa and/or lamina propria; 2: moderate diffuse edema in submucosa and/or lamina propria; 3: severe generalized edema in submucosa and/or lamina propria), presence of ulcerations (0: none; 1: very few, localized; 2: numerous; 3: extensive areas of ulceration with complete loss of villi), presence of inflammatory infiltrate (0: normal; 1: mild localized infiltrate; 2: mild generalized infiltrate; 3: severe generalized infiltrate), relative density of goblet cells (0: normal; 1: moderate diffuse increase in density; 2: moderate generalized increase in density; 3: generalized increase in density), and relative thickness of the muscle layers (0: normal; 1: mild increase; 2: moderate increase; 3: severe increase).

**Immunohistochemistry for rMCP-2 and rMCP-6 and MC Counts**

Immunodetection of rMCP-2 was done on jejunal sections following standard immunohistochemical procedures using a monoclonal antibody (MS-RM4, 1:500; Moredun Animal Health, Edinburgh, UK), as previously described by us (10).

For rMCP-6 detection, paraformaldehyde-fixed jejunal sections were rehydrated, and microwave antigen retrieval was performed (10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0; 2 cycles of 5 min, 800 W). After inhibition of endogenous peroxidases with 5% H2O2 for 40 min and incubation in rabbit serum for 1 h at room temperature, sections were incubated with goat anti-mouse cell tryptase antibody (sc-32473, overnight, 4°C; Santa Cruz Biotechnology, Santa Cruz, CA), followed by biotinylated rabbit anti-goat IgG (sc-2774, 1 h, room temperature, 1:200; Santa Cruz Biotechnology), and detection was performed with an avidin/peroxidase kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Antigen-antibody complexes were revealed using 3,3′-diaminobenzidine (SK-4100 DAB; Vector Laboratories), and sections were counterstained with hematoxylin. Specificity of the staining was confirmed by omission of the primary antibody.

Stained mucosal MCs (MMCs; rMCP-2 positive) were counted in at least 20 well-oriented villus-crypt units (VCU) per animal, at ×400 magnification, and expressed as MMCs per VCU. The total number of stained connective-tissue MCs (CTMCs; rMCP-6 positive) in the submucosa, external smooth muscle, and serosa areas was determined in two complete tissue sections of the jejunum for each animal (×600). CTMC counting was normalized for the surface area of submucosa, external smooth muscle, and serosa layers, as evaluated in digital images (Axioskop 40 microscope, Zeiss equipped with a digital camera, Zeiss AxioCam MRm, image analysis software: Zeiss Axiovision Release 4.8.1; Carl Zeiss, Jena, Germany). Cell counting and analysis of all data were performed in a blinded manner on coded slides, to avoid observer’s bias.

**PAR-2 Expression: Immunofluorescent Staining and Analysis**

Paraformaldehyde-fixed jejunal sections were rehydrated, and microwave antigen retrieval was performed (Citrate buffer, 10 mM, pH 6; 2 cycles of 5 min, 800 W). After blockade with 10% goat serum for 40 min at room temperature, the slides were incubated with rabbit anti-PAR-2 antibody (overnight, 4°C; 1:750; antibody A5). The A5 antibody is equivalent to the previously described B5 antiserum. A5 has been obtained in exactly the same way as B5, and both have the same specificity (2, 31, 32). Expression of PAR-2 was visualized using a Cy3-conjugated goat anti-rabbit secondary antibody (1 h, room temperature, 1:250, PA43004; GE Healthcare, Buckinghamshire, UK). The slides were coverslipped, and at least 16 representative photographs (original magnification ×1,000) were taken per animal with a Zeiss Axioskop 40 microscope equipped with a digital camera (Zeiss AxioCam MRm). The mean intensity of staining per square micrometer was measured employing the software Image J (National Institute of Health, Bethesda, MD). Changes in PAR-2 expression induced after *T. spiralis* infection were expressed as a percentage of the mean fluorescence intensity detected in the slides from the control group processed simultaneously. Specificity of the staining was confirmed by omission of the primary antibody. Analysis of all data was performed in a blinded manner to avoid observer’s bias.

**Using Chamber Studies**

**Measurement of electrophysiological parameters.** Electrophysiological parameters were measured in jejunal segments stripped of the outer muscle layers and myenteric plexus in Ussing chambers, as previously described by us (10). Tissues were bathed bilaterally with 5 ml of 37°C oxygenated Krebs buffer. The basolateral buffer contained 10 mM glucose, osmotically balanced with 10 mM mannitol in the apical buffer. Agar-salt bridges and electrodes were used to measure voltage and current across the epithelial layer. Electrical resistance was measured with a multimeter. Measurements were made under short-circuit conditions, at 37°C, under normal oxygen (95% O2, 5% CO2) at 0.9 MPa. Only segments with a transepithelial resistance greater than 1,000 Ω·cm2 were used. Staining and analysis of all data were performed in a blinded manner on coded slides, to avoid observer’s bias.
monitor potential difference (PD) and to inject the required short-circuit current ($I_{sc}$) to maintain a zero PD. A voltage step of 1 mV was applied every 5 min, and the change in $I_{sc}$ was used to calculate tissue conductance ($G$) by Ohm’s law. Tissues were allowed to stabilize for 15–25 min before baseline values were recorded. Data were digitized with an analog-to-digital converter, and measurements were recorded and analyzed with Acqknowledge computer software. $I_{sc}$ and $G$ were normalized for the exposed surface area (0.67 cm$^2$).

Electrical responses to PAR-2 activation. Changes in $I_{sc}$ ($\Delta I_{sc}$) in response to a single concentration of the PAR-2 activating peptide SLIGRL-NH$_2$ (3 × 10$^{-5}$ M; basolateral or apical addition) were assessed in control conditions and after $T$. spiralis infection. In addition, $\Delta I_{sc}$ in response to increasing concentrations of SLIGRL-NH$_2$ (10$^{-6}$–3 × 10$^{-5}$ M, 10-min interval between consecutive doses) added to the Ussing chamber (basolateral side) in a cumulative manner was studied. Some tissues were challenged with the control PAR-2 receptor-inactive reverse peptide LRGILS-NH$_2$ (3 × 10$^{-5}$ M) to assess specificity of the responses observed. In some experiments, to evaluate the involvement of the enteric nervous system, tissues were pretreated with tetrodotoxin (TTX, 10$^{-6}$ M), added to the basolateral side 10 min before SLIGRL-NH$_2$ (3 × 10$^{-5}$ M) addition. At the end of the experiments, a single dose of carbaryl (CCh, 10$^{-4}$ M) was added to the basolateral side of the chamber. CCh-induced $I_{sc}$ changes served to assess the viability of the tissues. $\Delta I_{sc}$ is expressed as the difference between the basal value before stimulation and the maximal response observed after treatment. All concentrations tested were selected based on preliminary studies and previously published data.

Assessment of epithelial permeability. Paracellular permeability was assessed by measuring mucosal to basolateral flux of fluorescein isothiocyanate-labeled dextran with an average molecular weight of 4 kDa (FD4; Sigma-Aldrich, St. Louis, MO) as previously described (10). After stabilization, FD4 was added to the mucosal reservoir to a final concentration of 2.5 × 10$^{-4}$ M. Basolateral samples (250 μl, replaced by 250 μl of appropriate buffer solution) were taken for subsequent fluorescence measurement against a standard curve. Readings are expressed as a percentage (%) of the total amount of FD4 added to the mucosal reservoir. FD4 fluxes were evaluated after the addition of the PAR-2-activating peptide, SLIGRL-NH$_2$, or the reverse-sequence PAR-2-inactive peptide, LRGILS-NH$_2$ (3 × 10$^{-5}$ M, basolateral or apical side), or trypsin (800 IU/ml, apical side). Ethylene glycol tetraacetic acid (EGTA) (8 × 10$^{-3}$ M), added simultaneously to each side of the Ussing chambers, was used as a positive control for induced enhanced permeability (34). Viability of the tissues was tested at the end of the permeability experiments by assessing responses to CCh (10$^{-4}$ M, basolateral side). Tissues with abnormal baseline values of electrophysiological parameters ($I_{sc}$, $G$, and PD) or with lack of response to CCh were considered damaged and were excluded. Overall, these represented less than 5% of the preparations tested.

Contractile Responses in Vitro

Full-thickness preparations of the jejunum were cut 1 cm long and hung for organ bath studies, oriented to record longitudinal muscle activity. Strips were mounted under 1 g tension in a 10-ml organ bath containing 37°C oxygenated Krebs buffer with glucose. One strip edge was tied to the bottom of the muscle bath and the other one to an isometric force transducer (Harvard VF-1 Harvard Apparatus, Holliston, MA) using silk 2/0 suture. Output from the transducer was fed to a PC through an amplifier. Data were digitalized using Data 2001 software (Panlab, Barcelona, Spain). Strips were allowed to equilibrate for about 1 h. To determine the spontaneous contractile activity, the preparation tone was measured for 5 min, and the area under the curve measured (in g). After this, maximal contractile responses to the PAR-2-activating peptide SLIGRL-NH$_2$ (6 × 10$^{-5}$ M) were assessed. Thereafter, bath solution was replaced, tissues were allowed to re-equilibrate (20 min), and then maximal changes in contractility induced by CCh (10$^{-4}$ M) were determined. In some experiments, contractile responses were studied in the presence of TTX (10$^{-6}$ M), added to the bath 30 min before addition of SLIGRL-NH$_2$ (6 × 10$^{-5}$ M). In some experiments, the PAR-2 reverse peptide LRGILS-NH$_2$ (6 × 10$^{-5}$ M) was also tested.

Solutions and Drugs

The composition of the Krebs buffer was (in mM): 10 glucose or mannitol, 115.48 NaCl, 21.90 NaHCO$_3$, 4.61 KCl, 1.14 NaH$_2$PO$_4$, 2.50 CaCl$_2$, and 1.16 MgSO$_4$ (pH 7.3–7.4). SLIGRL-NH$_2$ and LRGILS-NH$_2$ were obtained from Bachem (Essex, UK). Ketotifen fumarate salt, EGTA, trypsin, and CCh were acquired from Sigma-Aldrich. TTX was obtained from Latoxan (Valence, France). Stock solutions of SLIGRL-NH$_2$ (10$^{-2}$ M), LRGILS-NH$_2$ (10$^{-2}$ M), CCh (10$^{-1}$ M), and TTX (10$^{-4}$ M) in distilled water were used. Stock solutions of EGTA (1 M) and trypsin (80,000 IU/ml) were made up in NaOH 3 N and saline containing ascorbic acid (pH 5), respectively. In all cases, stock solutions were stored at −30°C, and further dilutions were performed in distilled water.

Statistical Analysis

All data are expressed as means ± SE. Comparisons between multiple groups were performed using a one-way ANOVA, a two-way ANOVA or a Kruskal-Wallis test, as appropriate, followed, when necessary, by a Newman-Keuls or a Dunns comparisons test. In all cases, results were considered statistically significant when $P < 0.05$.

RESULTS

Effects of $T$. spiralis Infection on Intestinal Histology

Histological alterations observed in the jejunum after $T$. spiralis infection were in concordance with our previously described findings (10, 41, 43). In brief, inflammatory infiltrate observed in the jejunum was more severe on day 14 postinfection (score: control: 0.0 ± 0.0 vs. day 14 postinfection: 1.47 ± 0.27; $P < 0.001$; $n = 4–12$), at which time it was accompanied by submucosal edema (score: control: 0.0 ± 0.0 vs. day 14 postinfection: 0.5 ± 0.1; $P < 0.001$; $n = 4–12$) and epithelial damage (mainly villus atrophy and crypt hyperplasia; score: control: 0.25 ± 0.08 vs. day 14 postinfection: 2.53 ± 0.28; $P < 0.01$; $n = 4–12$). By day 30 postinfection, the intestinal mucosa had essentially recovered its normal appearance although a slight inflammatory response was still present (inflammatory infiltrate score: 0.31 ± 0.16; $n = 8$; $P > 0.05$ vs. control). Hyper trophy of external muscular layers was visible during the entire postinfectious period (score: control: 0.16 ± 0.09 vs. day 14 postinfection: 2.40 ± 0.37 or day 30 postinfection: 1.62 ± 0.18; both $P < 0.001$ vs. control; $n = 4–12$).

Effects of $T$. spiralis Infection on PAR-2 Expression

In control conditions, PAR-2-like immunoreactivity was present on the apical surface of epithelial cells and on the submucosal and myenteric plexuses (Fig. 1), in accord with previous observations (21). On epithelial cells of infected rats, PAR-2 immunoreactivity was diminished by 35% ($P < 0.05$) on day 14 postinfection, being partially restored on day 30, although expression was still lower (by 20%) than in control conditions (Fig. 1). On the other hand, PAR-2-like immunoreactivity on submucosal ganglia showed a progressive increase during the postinfectious phase, reaching the highest intensity at 30 days postinfection. PAR-2-like immunoreactivity on...
myenteric ganglia was similar across groups although it tended to be higher on day 14 after *T. spiralis* infection (Fig. 1).

**Effects of *T. spiralis* Infection and PAR-2 Activation on Transepithelial Ion Transport**

At days 14 and 30 postinfection, basal $I_{sc}$ was not affected, whereas an increase in $G$ and a decrease in PD were observed (Table 1). None of these parameters was affected by the addition of TTX to the basolateral side (Table 1).

In control conditions, basolateral, but not apical, application of SLIGRL-NH$_2$ led to a rapid, concentration-related increase in $I_{sc}$ (Fig. 2A). In contrast, LRGILS-NH$_2$, the PAR-2-inactive form of the peptide, or vehicle (distilled water) applied to either the basolateral or the apical side did not affect $I_{sc}$ (Fig. 2C). Similarly, the apical application of SLIGRL-NH$_2$ or the apical or basolateral application of LRGILS-NH$_2$ in jejunal samples from infected animals was ineffective (data not shown).

In either controls or infected animals, basolateral application of SLIGRL-NH$_2$ induced concentration-dependent changes in electrogenic ion transport (Fig. 2, A and B). Cumulative concentrations of SLIGRL-NH$_2$ did not result in tissue desensitization, as $I_{sc}$ responses to the highest concentration tested were

<table>
<thead>
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<th>Animals, number</th>
<th>Jejunal Sheets, number</th>
<th>$I_{sc}$, $\mu$A/cm$^2$</th>
<th>PD, mV</th>
<th>$G$, ms/cm$^2$</th>
</tr>
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<tr>
<td>Control</td>
<td>18</td>
<td>45–48</td>
<td>$-28.12 \pm 1.89$</td>
<td>$1.17 \pm 0.08$</td>
</tr>
<tr>
<td>Control + TTX</td>
<td>9</td>
<td>9</td>
<td>$-31.73 \pm 5.80$</td>
<td>ND</td>
</tr>
<tr>
<td><em>T. spiralis</em> (day 14)</td>
<td>6</td>
<td>23</td>
<td>$-34.35 \pm 1.74$</td>
<td>$0.94 \pm 0.06^{*}$</td>
</tr>
<tr>
<td><em>T. spiralis</em> (day 14) + TTX</td>
<td>6</td>
<td>6</td>
<td>$-35.95 \pm 5.49$</td>
<td>ND</td>
</tr>
<tr>
<td><em>T. spiralis</em> (day 30)</td>
<td>18</td>
<td>42–43</td>
<td>$-28.45 \pm 2.06$</td>
<td>$0.75 \pm 0.04^{‡}$</td>
</tr>
<tr>
<td><em>T. spiralis</em> (day 30) + TTX</td>
<td>7–8</td>
<td>7–8</td>
<td>$-34.99 \pm 4.28$</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are means ± SE of the number of jejunal sheets indicated. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ vs. control group. *T. spiralis*, *Trichinella spiralis*; $G$, conductance; $I_{sc}$, short-circuit current; ND, not determined; PD, potential difference; TTX, tetrodotoxin.
Fig. 2. Effects of basolateral activation of PAR-2 on epithelial short-circuit current (Isc). A: representative tracings showing changes in Isc induced by SLIGRL-NH2 (3 × 10⁻⁵ M) in the different experimental groups. Note the biphasic response elicited by PAR-2 activation and the changes associated to the acute phase of inflammation (day 14 postinfection), with a clear recovery by day 30 postinfection. B: cumulative concentration-response curves for the PAR-2-activating peptide (SLIGRL-NH2), with and without preincubation with tetrodotoxin (TTX). Data represent changes in Isc (ΔIsc in μA/cm²) over basal values and are means ± SE of 6–12 mucosal sheets from 6–11 animals per group. **p < 0.01, ***p < 0.001 vs. respective group at day 30 postinfection [T. spiralis (day 30) or T. spiralis (day 30) + TTX]. C: changes in Isc (ΔIsc in μA/cm²) induced by the PAR-2-activating peptide (SLIGRL-NH2, 3 × 10⁻⁵ M), the control PAR-2 receptor-inactive reverse peptide (LRGILS-NH2, 3 × 10⁻⁵ M), or vehicle (distilled water) added either to the apical (A) or the basolateral (BL) side of the Ussing chamber, in control conditions. Data are means ± SE of 6–11 mucosal sheets from 4–11 animals per group. ***p < 0.001 vs. all other experimental groups.

Effects of T. spiralis Infection and PAR-2 Activation on Paracellular Permeability to Fluorescent Tracers

Mucosal to basolateral passage of FD4 across jejunal segments was time dependent in all groups. However, permeability to FD4 was significantly increased in the jejunum of previously infected animals. At the end of the 1-h experimental time, transepithelial passage of FD4 was increased by 63.7 ± 11.7% and 104.8 ± 30.0% in infected animals, at days 14 and 30 postinfection, respectively, over control values in noninfected animals (both P < 0.001 vs. noninfected controls; Fig. 3A).

Neither apical nor basolateral addition of SLIGRL-NH2 affected paracellular permeability to FD4 in control conditions or on day 30 postinfection (Fig. 3, B and C). Mucosal application of EGTA, but not trypsin, significantly increased FD4

similar after single or cumulative application both in controls (single dose of 3 × 10⁻⁵ M: 51.76 ± 8.15 μA/cm² vs. 3 × 10⁻⁵ M in cumulative dose-response curves: 52.78 ± 6.50 μA/cm²; P > 0.05; n = 8–10) and in rats on 30 days postinfection (single dose of 3 × 10⁻⁵ M: 28.47 ± 5.52 μA/cm² vs. 3 × 10⁻⁵ M in cumulative dose-response curves: 35.36 ± 4.50; P > 0.05; n = 7–12). SLIGRL-NH2-induced changes in Isc were transitory and rapid, beginning within 1 min after the application of the peptide, reaching a peak within 2–3 min, and recovering stable Isc values during the subsequent 3 min. At the higher concentrations tested (10⁻⁴ and 3 × 10⁻⁵ M), Isc changes were biphasic, with a primary response of low magnitude occurring within the first minute after application of the peptide, followed by a second peak of higher magnitude, within the 2–3-min period after application of the peptide (Fig. 2A). The magnitude and time course of the responses to PAR-2 activation were significantly altered during the early postinfectious phase. At day 14 postinfection, maximal responses to PAR-2 activation were reduced by 62% (P < 0.001 vs. control), affecting mainly the second phase of the responses (Fig. 2, A and B). A significant recovery in both the magnitude and the time course of the responses was observed on day 30 compared with day 14 although increases in Isc were still attenuated (by 33%) with respect to the control group (Fig. 2, A and B). Regardless of the group considered, responses to SLIGRL-NH2 were not affected by TTX pretreatment (Fig. 2B).
flux in control conditions (Fig. 3, D and E). The inactive peptide LRGILS-NH$_2$ did not affect epithelial permeability.

**Effects of *T. spiralis* Infection and PAR-2 Activation on Jejunal Contractility**

Spontaneous jejunal contractility was increased at day 14 postinfection, returning to control values by day 30. Neural blockade with TTX inhibited spontaneous contractility by 32%, 23%, and 48% in control conditions and at days 14 and 30 postinfection, respectively (Fig. 4, A and B).

In all cases, addition to the bath of the PAR-2 agonist SLIGRL-NH$_2$ (6 $\times$ 10$^{-5}$ M) induced a contractile response, reaching a maximum at 3–4 min after the addition of the activating peptide and lasting for about 3 min. In about 50% of the muscle strips in controls and on day 30 postinfection and in all strips from day 14 postinfection, during the most acute phase of the inflammatory response, a transient relaxation was observed during the first minute after the addition of the peptide, before the occurrence of the contractile response. Moreover, the magnitude of the initial relaxation to SLIGRL-NH$_2$ was enhanced (by 14-fold) on day 14 postinfection, when inflammation was at its peak (Fig. 4, C and D). No response was observed following exposure to the reverse peptide LRGILS-NH$_2$.

The increase in tension generated after CCh addition was increased after *T. spiralis* infection, with responses that were comparable on days 14 and 30 postinfection (Fig. 4E). Responses to either PAR-2 activation or CCh were insensitive to TTX (data not shown).

**Effects of *T. spiralis* Infection on MC Counts**

In control rats, MMCs were located in the mucosa and the submucosa (Fig. 5A), whereas CTMCs were only occasionally observed in the serosa (Fig. 5B). Infection with *T. spiralis* was associated with an infiltration of MMCs (Fig. 5, C and E) and CTMCs in the jejunum (Fig. 5, D and F). During the postinfectious stages, CTMCs were observed in the serosal surface but also infiltrating the external muscle layers (Fig. 5, D and F).

**Effects of Ketotifen on Intestinal EBF and Motility**

Ketotifen treatment, per se, did not affect basal jejunal electrical parameters or contractility or the responses to PAR-2 activation (Fig. 6). Likewise, postinfectious-associated changes in PAR-2-mediated control of EBF and contractility were not affected by ketotifen (Fig. 6). In infected animals, spontaneous contractility was increased by 111.6% and 23.9% over control values at 14 and 30 days postinfection (n = 10–12 jejunal strips), respectively. In ketotifen-treated animals, this increase in spontaneous contractility was attenuated to 22.1 ± 14.7% and 23.9 ± 14.2% over control values at 14 and 30 days postinfection (n = 10–12 jejunal strips), respectively. However, statistical significance was not reached, likely because of variability within the data.
DISCUSSION

The present study describes changes in PAR-2 function and expression related to the early and late phases of a rat model of postinfectious gut dysfunction. Results obtained show alterations in PAR-2 epithelial expression and PAR-2-dependent control of secretomotor and contractile responses during the early phase (day 14 postinfection), associated with a severe inflammation. In contrast, a clear trend toward normality in PAR-2 epithelial levels and PAR-2-mediated responses was observed during the late phase of the inflammatory response (day 30 postinfection). However, an increase in PAR-2 expression was observed in the submucosal ganglia in the late postinfectious phase, when the main finding is a persistent MC infiltrate. To our knowledge, this is the first study comparing PAR-2 expression and control of EBF and contractility in different phases of a postinfectious gut dysfunction model.

We evaluated how PAR-2 activation could modulate jejunal permeability in the rat, as previously shown in the colon. However, in contrast with observations done with colon preparations, we found that the PAR-2-activating peptide SLIGRL-NH$_2$ did not affect jejunal permeability in our conditions. This lack of effect on permeability was not associated with an absence of activity of the peptide because SLIGRL-NH$_2$ induced significant secretory and contractile responses of the tissue at similar concentrations. Furthermore, this result was not due to a lack of sensitivity of the Ussing chambers because changes in permeability could be detected when present, such as after infection or EGTA addition (10, 34). Recent data point to differences in epithelial barrier modulation by the PAR-2-activating peptide and endogenous agonists with enzymatic activity (39). Therefore, we tested whether trypsin, as an endogenous activator of PAR-2, was capable of modulating intestinal permeability. However, trypsin, like SLIGRL-NH$_2$, was not able to increase permeability. These data suggest that PAR-2 activation is not able to modulate paracellular permeability in the rat jejunum, thus pointing to a differential PAR-2-mediated control of permeability between the small intestine and the colon.

In contrast to its lack of effect on permeability, PAR-2 activation clearly influenced jejunal motility and hydroelectro-
lytic transport. Overall, contractile responses to PAR-2 activation were biphasic, with a transient relaxation followed by a contraction. In control conditions, the contractile phase predominated over the relaxation, in agreement with the PAR-2-mediated enhancement of intestinal motility observed under in vivo conditions (15). Similar PAR-2-induced biphasic responses were also observed when assessing hydroelectrolytic transport. PAR-2-mediated changes in $I_{sc}$ would reflect ionic movement to the lumen, leading to the secretion of water (23, 47). Effects of PAR-2 activation were short lasting, in agreement with that previously described in similar in vitro systems (36, 47). Nevertheless, these effects might have functional implications in vivo, particularly in conditions in which PAR-2 can be tonically activated, such as during states of MC infiltration/activation (23, 47), likely leading to sustained functional changes. Therefore, it is feasible that PAR-2-mediated increased motility and luminal secretion would contribute to a “protective” response, controlling penetration and interaction of luminal antigens with the epithelium and bacterial overgrowth in physiological conditions (3, 27). In addition, these PAR-2-mediated defensive responses are apparently induced by direct effects on the epithelium and the muscle, as the modulation of secretion and motility by SLIGRL-NH$_2$ was TTX independent (25, 26, 47). Nevertheless, we cannot rule out

Fig. 5. Jejunal mucosal and connective tissue mast cells. A, C, and E: representative microphotographs showing rat mast cell protease-2 (rMCP-2)-immunopositive cells (corresponding to mucosal MCs, MMCs) in the jejunal mucosa and submucosa of a control (A) and T. spiralis-infected rats at days 14 (C) and 30 postinfection (E) ($\times$100 magnification). B, D, and F: representative microphotographs showing rMCP-6-immunopositive cells (corresponding to connective-tissue MCs, CTMCs; arrow heads) in the jejunal muscular layers and serosa of a control ($\times$100) (B) and a previously infected animal, at day 30 postinfection ($\times$200 (D) and $\times$600 magnification (F)). Bar graphs at the bottom show the quantification of MMCs (left) and CTMCs (right). MMC quantification is represented as number of rMCP-2-positive cells per villus-crypt unit (VCU). Data correspond to means $\pm$ SE, from 6 – 13 animals per group. **$P < 0.01$, ***$P < 0.001$ vs. control group. CTMC quantification is presented as number of rMCP-6-positive cells per surface area (mm$^2$) of submucosa, muscularis externa, and serosa layers. Data correspond to means $\pm$ SE from 5 – 6 animals per group. **$P < 0.01$ vs. control group.
the participation of specific neurotransmitters in the different phases of PAR-2-mediated responses, as previously described in the rat colon (25). Further studies are needed to characterize the underlying mechanisms generating PAR-2-mediated biphasic secretory and motor responses in the jejunum.

On day 14 postinfection, corresponding to the intestinal phase of T. spiralis infection, increased intestinal spontaneous motility and permeability could contribute to the expulsion of the nematode (17, 24). In this early postinfectious phase, in agreement with observations in nematode-infected mice, PAR-2 activation induced a predominant relaxation of the smooth muscle, in contrast to the responses described above for control conditions. At the same time, TTX-independent reductions in secretory responses to SLIGRL-NH₂ point to a postinfectious epithelial dysfunction, rather than to a neurally mediated effect. Indeed, immunostaining studies suggest that PAR-2 dysfunction is originated by a loss of receptors in the epithelial surface after nematode infection (36 and present observations). This loss could be related to enhanced levels of proteinases, including those produced by the parasites, in infected animals, which would increase PAR-2 activation and internalization, leading to a reduced receptor presence on the cell surface (9, 20, 36, 44). Changes in motor and epithelial effects of PAR-2 activation associated with the early postinfectious state might have a dual role. On the one hand, these changes might be beneficial, minimizing the loss of liquid during diarrheic-type states. On the contrary, reduced motor and secretory responses may delay parasite expulsion and facilitate its interaction with the epithelium and penetration. Moreover, these changes could favor host-microbial interactions and the penetration of luminal antigens and/or bacterial translocation, thus contributing to the inflammatory process observed at the histopathological level.

Interestingly, by day 30 postinfection, both basal motility and contractile responses to SLIGRL-NH₂ were normalized. Therefore, changes in PAR-2-mediated motor responses were only observed in association with altered basal motility and would contribute to normalize the intestinal transit. As a consequence, our results support a modulatory role of PAR-2 activation in intestinal motility. Regarding PAR-2-mediated secretory responses, although a significant recovery was observed during the late postinfectious phase, epithelial barrier alterations had a long-term persistence, even after parasite expulsion from the intestinal lumen (37). This change was associated with an incomplete recovery of PAR-2 expression in the epithelial border and an increased PAR-2 expression in the submucosal ganglia. In that respect, PAR-2-activating proteinases, such as those released by MCs, could condition enteric nervous system excitability and, in turn, secretion, motility, and visceral sensation (29). However, as previously discussed for control conditions and the early postinfectious phase, motility and secretory responses to SLIGRL-NH₂ were insensitive to TTX on day 30 postinfection. Therefore, PAR-2 dysfunction seems to contribute to the chronic epithelial remodeling observed in the late postinfectious phase (10). Taking into consideration the rapid turnover of the epithelial cells lining the gut (12), other factors, in addition to the presence of the parasite, would condition epithelial PAR-2 expression and function in the late postinfectious stages.

Several endogenous and exogenous proteinases, other than those produced by the parasites, are candidates to modulate postinfectious changes in PAR-2 expression and functionality. In that respect, one of the main features of T. spiralis infection is a persistent MC infiltration, an important source of endogenous proteinases (10, 28, 35). In particular, MC-derived tryptase has been suggested as one of the main endogenous activators of PAR-2 (1). However, tryptase per se has been found to be unable to activate PAR-2 on its own in an in vitro setting, presumably due to the glycosylation of the target receptor proteinase activation site (6). In our case, the infiltration of CTMCs, expressing tryptase, in the smooth muscle and the serosa supports their potential role in the functional changes observed. Thus, although MC-derived proteinases may well contribute to the dysfunction of the tissue, it is possible that the effects are unrelated to a modulation of PAR-2. Supporting this view, increasing data implicate MMCs in the EBF and motility changes observed in inflammatory and functional gut disorders (10, 24, 30, 35). Our suggestion that...
this impact of MC-derived enzymes is not via PAR-2 is supported by our data observing the effects of the MC stabilization with ketotifen. Because ketotifen treatment did not affect the PAR-2-mediated effects, postinfectious PAR-2 alterations seem to be independent of a functional interplay between MCs and PAR-2. This conclusion would explain why postinfectious PAR-2 alterations were mostly resolved by day 30 postinfection despite the persistent MC hyperplasia. These results are unlikely to be due to a lack of effect of ketotifen because our protocols and doses are known to block MC proteinase release effectively, preventing gut dysmotility in T. spiralis-infected rats, as shown in previous studies under similar experimental conditions (14, 35), or to prevent postoperative ileus in both animals and humans (8, 42). Indeed, in our experiments, ketotifen reduced spontaneous hypermotility by 78% in infected animals, suggesting an effective blockade of MC degranulation and confirming a role for MCs in the intestinal hypermotility that characterizes this model. Although pharmacological effects of ketotifen are primarily associated to a stabilization of MCs, from the present observations, we cannot discard a contribution of other targets also affected by ketotifen, such as H1 receptors.

In summary, we show that postinfectious intestinal alterations in PAR-2-induced secretory and contractile responses are mainly associated with the early phase of gut dysfunction and related to a reduction in the receptor expression in the epithelial border. In contrast, most changes in PAR-2 are resolved in the late postinfectious phase although MC hyperplasia, involving both MMCs and CTMCs, persists. This result, together with the lack of influence of MC stabilization, suggests that MC mediators are not responsible for PAR-2-mediated responses in this model. Therefore, further studies assessing alternative activators of PAR-2, other than MC proteinases, are necessary to understand the underlying mechanisms involved in PAR-2 alterations related to gastrointestinal diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: J.A.F.-B., V.M., and P.V. conception and design of research; J.A.F.-B., V.M., and P.V. performed experiments; J.A.F.-B. and V.M. analyzed data; J.A.F.-B., V.M., and P.V. interpreted results of experiments; J.A.F.-B. and V.M. performed experiments; J.A.F.-B. and V.M. contributed reagents/analytic tools; J.A.F.-B., V.M., and P.V. wrote paper; J.A.F.-B., V.M., and P.V. approved final version of manuscript.

REFERENCES

22. Lee JW, Park JH, Park DI, Park JH, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI. Subjects with diarrhea-predominant IBS have increased...


