The Nitric Oxide Donor LA-419 [S-(6-Nitro-oxi-hexahydrofuro[3,2-b]furan-3-1-il)thioacetate] Prevents Intestinal Dysmotility, Bacterial Translocation, and Inflammation in a Rat Model of Enteritis

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ABSTRACT

Indomethacin induces a chronic model of inflammatory bowel disease (IBD) characterized by spontaneous relapses of inflammation, bacterial translocation, and long-lasting motor disturbances derived from cyclical up-regulated inducible nitric-oxide synthase (iNOS) and sustained down-regulated neuronal NOS (nNOS). The aims of this study were to evaluate whether LA-419 [S-(6-nitro-oxi-hexahydro-furo[3,2-b]furan-3-1-il)thioacetate], a NO-donor drug, could re-establish the normal expression of NOS and, hence, prevent the development of intestinal dysmotility, bacterial translocation, and relapses of inflammation associated to this model. Enteritis was induced in rats by administration of indomethacin with and without treatment with a novel NO-donor: LA-419 (0.5 mg/ml in the drinking water). Inflammatory reaction was evaluated by measuring blood leukocytes, serum tumor necrosis factor, and tissue myeloperoxidase. Intestinal motor activity was evaluated using strain-gauges. Ileal expression of iNOS and nNOS mRNA was determined by reverse transcription-polymerase chain reaction. Bacterial translocation was evaluated in cultures from mesenteric lymph nodes. The indomethacin-induced acute inflammatory reaction was associated with a rise in blood leukocytes and tumor necrosis factor. In the chronic stage, blood leukocyte monitoring allowed the selection of animals in active and inactive phases. Active phase was associated with iNOS up-regulation, high myeloperoxidase levels, hypomotility, and bacterial translocation. In contrast, inactive phase was associated with hypermotility and absence of bacterial translocation. LA-419 treatment restored nitric-oxide synthase isoenzyme expression and prevented the oscillation of both inflammatory and motor parameters that could be cyclically observed in inflamed rats. LA-419 also prevented intestinal dysmotility, bacterial translocation, and relapses of intestinal inflammation. LA-419 might be a novel therapeutic approach to prevent acute inflammatory relapses in patients with IBD.

In the gastrointestinal tract, nitric oxide (NO) levels result from the activity of both constitutive synthase and inducible isoforms of NO synthase (iNOS) (Alican and Kubes, 1996). NO displays several functions in the healthy state, such as a regulator of the gastrointestinal motility and mucus secretion (Whittle et al., 1990; Martin et al., 2001). However, NO has also been implicated in a wide number of chronic inflammatory pathologies, including idiopathic inflammatory bowel disease (IBD) (Dijkstra et al., 1998; Shah et al., 2004). In this case, an overproduction of NO via iNOS up-regulation has been found in both ulcerative colitis and Crohn's disease (Kimura et al., 1997), a feature that has also been reproduced in different experimental models of IBD (Kubes and McCafferty, 2000). It is interesting that a concomitant down-regulation of nNOS has also been observed during inflammation (Takahashi, 2003). This feature has been found in different experimental models, such as the dextran sulfate sodium-induced colitis (Mizuta et al., 2000), in the 2,4,6-trinitrobenzene sulfonic acid-induced colitis (Depoortere et al., 2002), and in the mucosa of patients with ulcerative colitis (Menchen et al., 2004). Taken together, these data indicate a strong relationship between intestinal inflammation and dysregulation of both isoforms of NO synthase,

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ABBREVIATIONS: NO, nitric oxide; IBD, inflammatory bowel disease; LA-419, S-(6-nitro-oxi-hexahydro-furo[3,2-b]furan-3-1-il)thioacetate; iNOS, inducible nitric-oxide synthase; MPO, myeloperoxidase; NF-κB, nuclear factor κB; nNOS, neuronal nitric-oxide synthase; NOS, nitric-oxide synthase; PCR, polymerase chain reaction; L-NNA, Nω-nitro-L-arginine; AUC, AUC, area under the curve; CCK, cholecystokinin.
suggesting that NO plays a fundamental role in the pathophysiology of IBD.

Up-regulated iNOS generates large amounts of NO that can increase gut permeability, induce apoptosis, stimulate epithelial secretion, promote hypomotility and bacterial translocation, and contribute to intestinal injury via the production of cytotoxic peroxynitrite (Grisham et al., 2002). However, iNOS-derived NO can also kill bacteria, block apoptosis, and reduce inflammation by inhibiting activation of NF-κB (Kubes, 2000). These contradictory effects have been demonstrated in pharmacological studies using iNOS inhibitors, showing either protective (Rachmilewitz et al., 1995) or the detrimental (Dikopoulos et al., 2001) role of NO in the inflammatory state. On the other hand, because NO produced by nNOS is implicated in the maintenance of mucosal integrity of the gastrointestinal tract (Whittle and Lopez-Belmonte, 1993), down-regulation of this isoform might contribute to the bacterial translocation observed in IBD.

It is well known that complex relationships exist between NO isoforms. NO derived from nNOS down-regulates iNOS expression via the suppression of NF-κB activation (Qu et al., 2001). In contrast, release of large amounts of NO by up-regulated iNOS has been reported to inhibit NO-expressing neurons (Mizuta et al., 2000). Furthermore, exogenous administration of NO-releasing compounds regulates iNOS and/or nNOS expression on different cell types (Assreuy et al., 1993; Kurjak et al., 1999; Zandecki et al., 2006). Thus, the resulting action of NO in IBD could depend on changes in the nNOS/iNOS balance, which could be a key feature in the appearance of spontaneous relapses observed in patients and, hence, in the perpetuation of the disease.

Recently, we have obtained a new experimental model of enteritis in the rat that reproduces the oscillation of active and inactive phases characteristic of IBD (Porras et al., 2004; Silva et al., 2006). Rats with high levels of proinflammatory markers (active phase) show intestinal hypomotility associated with bacterial translocation. By contrast, animals with lower levels of proinflammatory markers (inactive phase) have intestinal hypomotility and absence of bacterial translocation (Porras et al., 2006a). It is interesting that these opposite patterns are associated with a dysregulated expression of NOS isoenzymes; a sustained down-regulation of nNOS exists through the inflammatory state (in both active and inactive phases), with iNOS up-regulation occurring only during the active phase (Porras et al., 2006b). Thus, this model can be useful to study the implication of possible changes in nNOS/iNOS balance in the relapses seen in IBD patients.

Our hypothesis is that an imbalance between nNOS and iNOS, caused by high NO levels produced during early inflammatory stage, can play a fundamental role in the course of IBD. Thus, treatment with NO-releasing compounds would result in a maintenance of physiological levels of NO, preventing the nNOS/iNOS imbalance and, hence, avoiding the appearance of relapses observed in IBD patients. The NO-donor used in this study was LA-419, a novel NO donor that has been designed to treat clinical conditions associated with a reduced bioavailability of NO (Vilahur et al., 2004; Hernandez et al., 2005; Serrano et al., 2007). The aim of the study was to evaluate whether the treatment with LA-419 can re-establish the normal NO bioavailability and its consequences on intestinal dysmotility, bacterial translocation, and relapses of inflammation seen in our experimental model.

### Materials and Methods

#### Animals

Male Sprague-Dawley rats weighing 300 to 350 g at the beginning of experiments were obtained from Charles River (Lyon, France). Animals were housed individually under pathogen-free conditions in a temperature- (20–21°C) and humidity-controlled (55–60%) room with a 12-h light/dark cycle and provided with standard rodent chow and water ad libitum. All experimental protocols were carried out under the supervision of the Ethical Committee of the Universitat Autònoma de Barcelona (Barcelona, Spain).

#### Experimental Model

Intestinal inflammation was induced by administration of two injections of indomethacin (7.5 mg/kg s.c.) 48 h apart, as described previously (Porras et al., 2004, 2006b). This model shows a spontaneous alternation of active and inactive phases of inflammation, where active phases are characterized by a generalized hypomotility, bacterial translocation, and an increase of proinflammatory markers (blood leukocytes, TNF-α, and myeloperoxidase (MPO)), whereas the quiescent periods are characterized by hypermotility, absence of bacterial translocation, and reduction of inflammatory parameters. Blood samples were taken every 3 days to monitor blood leukocytes levels, allowing the selection of animals during either active (high blood leukocytes, 19,614 ± 222 cells/mm³) or inactive (low blood leukocytes, 15,369 ± 259.7 cells/mm³) phases.

#### Experimental Groups

The following groups were established: 1) inflamed control group, receiving two injections of indomethacin (7.5 mg/kg s.c.) 48 h apart; 2) control group, receiving saline solution; 3) LA-419 (LACER SA, Barcelona, Spain) control group, receiving LA-419 in drinking water for 18 days (0.5 mg/ml) 4) inflamed LA-419-pre-treated group, receiving indomethacin as inflamed control group and LA-419 (0.5 mg/ml) from 5 days before the administration of indomethacin until the end of the study; and 5) inflamed LA-419-treated group, receiving indomethacin as inflamed control group and LA-419 (0.5 mg/ml) from 2 days after the first injection of indomethacin, when inflammation was already established, until the end of the study. Experiments were performed at day 15 ± 3 after the first injection of indomethacin (inflamed control group), being animals selected according to their blood leukocyte levels as in active or inactive phase. In the rest of groups, experiments were performed at day 18.

#### Animal Monitoring and Samples Collection

The following parameters were monitored daily in all groups: body weight, food consumption, and water consumption. Blood samples were taken every 2 to 3 days between 10:00 AM and 11:00 AM to monitor blood leukocytes by using a Neubauer chamber. TNF-α concentration was measured at days 2, 4, 10, and 15 using a specific enzyme-linked immunosorbent assay (rat TNF-α; Biosource International, Camarillo, CA) with a minimal detectable concentration of 4 pg/ml. Rats were killed by decapitation after light anesthesia. Small bowel was collected and cleaned by flushing with buffered saline. Intestinal segments were then fixed in 4% buffered formalin or quick-frozen in liquid nitrogen and kept at −80°C for further analysis.

#### Morphology and Microscopic Score of Inflammation

Tissue samples previously fixed in buffered formalin were embedded in paraffin and processed for histopathology according to standard procedures. Microscopic inflammation was assessed by an observer blinded to rat treatment in triple sections from each rat as described previously (Silva et al., 2006). The parameters evaluated were the following: 1) mucin alterations, scoring 0 for normal number of goblet cells and 1 for abnormal cell number (normality was defined as 16–28 goblet cells/120 epithelial cells); 2) inflammatory cell infiltration, scoring 0 for the absence of infiltration and 1, 2, and...
3 for the presence of infiltration in the lamina propria, submucosa, and muscular layers, respectively; 3) crypt architectural irregularities, scoring 0 for normal crypts an 1 for the presence of abnormal crypts; and 4) surface epithelial integrity, scoring 0 for normal morphology, 1 for discontinuity of the epithelial layer, and 2 for the presence of ulcers. The sum of all the parameters generated a score of 0 to 7 for histological inflammation.

**Tissue Myeloperoxidase Determination**

Ileal tissue samples were powdered, weighted, and homogenized in lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerine, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, and 28 µg/ml aprotinin, pH 7.4). Homogenized tissue samples were centrifuged at 1000 g for 15 min at 4°C. Supernatant was removed and stored at -80°C until use. Myeloperoxidase (MPO) concentration was determined using a specific enzyme-linked immunosorbent assay (HyCult Biotechnology, Uden, The Netherlands), with a minimal detectable concentration of 1 ng/ml.

**Reverse Transcription-PCR Studies**

Total RNA was extracted from ileal samples using RNAwiz (Ambion, Madison, WI) and treated with DNA-free (Ambion) for 30 min at 37°C to remove any genomic DNA contamination. cDNA was synthesized from 5 µg of total RNA in a reaction mixture of 50 µl containing 0.5 µg of oligo 18(dT) primer (Ambion), 2 mM dNTP (Ecogen, Barcelona, Spain), and 10 units of Moloney murine leukemia virus reverse transcriptase (Ambion). The resultant cDNA was amplified in a total volume of 50 µl, with 1 unit of Taq DNA polymerase (Ambion), 2 mM dNTP mixture, and 0.5 µM primers (Proligo; Sigma Chemical), as described previously (Porras et al., 2006b). Amplified products were electrophoresed in 1.5% agarose gel in Tris-acetate EDTA buffer, stained with ethidium bromide, photographed under ultraviolet light, and quantified using image-analyzing software (Quantity-One, Bio-Rad Laboratories, Hercules, CA). For semiquantification, the ratio of the optical density of each PCR product and glyceraldehyde-3-phosphate dehydrogenase was determined.

**Bacterial Translocation**

Detection of viable enteric bacteria in mesenteric lymph nodes is considered as a key evidence of bacterial translocation from the intestine (Mainous et al., 1991). Mesenteric lymph nodes from the ileocecal region were removed under sterile conditions from rats pretreated with LA-419 (Laboratorios Lacer, Barcelona, Spain) was dissolved in drinking water at a concentration of 0.5 mg/ml that allowed dosing the product at 30 mg/kg/day. CCK-8 (Peptide Institute, Osaka, Japan) was diluted in 1% NaHCO3 solution to a concentration of 10−4 M and in saline solution to work concentration. L-NNA (Sigma Chemical) was diluted in saline solution.

**Statistical Analysis**

Data are expressed as means ± S.E.M. Statistics were performed with Prism version 3.0 software (GraphPad Software Inc., San Diego, CA). Differences between groups were compared using one-way or two-way analysis of variance and Bonferroni’s post-hoc analysis. Single comparisons were performed using Student’s t test. Results from bacterial translocation study were compared using the chi-square test. In all cases, the results were considered to be statistically significant when P < 0.05.

**Results**

**Animal Monitoring**

A significant decrease in food consumption and body weight was observed for the first 5 days after induction of inflammation in all groups receiving indomethacin. However, the weight loss was comparably less in the inflamed group pretreated with LA-419 (P < 0.01). After the 1st week, an increase in both parameters was observed. However, despite food consumption returning to normal values, body weights remained 10% lower (inflamed LA-419-pretreated group) and 15% lower (inflamed control group and inflamed LA-419-treated group) than controls (P < 0.01). The administration of LA-419 to noninflamed animals did not modify either food consumption or body weight. Tables 1 and 2 show values obtained at days 4 and 15, respectively.

**Blood Leukocytes and TNF**

In all groups receiving indomethacin, body weight loss was paralleled to an increase in blood leukocytes and plasmatic TNF. However, the rise of both parameters was lower in the two inflamed groups receiving LA-419 (as preventive and therapeutic treatment) compared with the inflamed control group (Table 1, P < 0.01). Moreover, both preventive and therapeutic LA-419 treatments were effective in preventing the characteristic oscillation of blood leukocytes and TNF seen in animals with indomethacin-induced enteritis (Table 2).

**Histological Study**

As described previously (Porras et al., 2004), administration of indomethacin induced an important acute inflammatory reaction in the small intestine, especially in the distal jejunum and ileum. The inflammatory reaction at day 4 was...
TABLE 1
Effect of LA-419 on food consumption, body weight, blood leukocytes, and TNF during the acute phase (day 4) of indomethacin-induced enteritis in rats
Data are expressed as means ± S.E.M. (n = 12).

<table>
<thead>
<tr>
<th>Group</th>
<th>Food Consumption</th>
<th>%</th>
<th>Leukocytes</th>
<th>TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.3 ± 2.6</td>
<td>7.2 ± 1.7</td>
<td>11,713 ± 1059</td>
<td>&lt;4</td>
</tr>
<tr>
<td>LA-419</td>
<td>26.2 ± 2.2</td>
<td>6.9 ± 1.5</td>
<td>12,917 ± 1059</td>
<td>&lt;4</td>
</tr>
<tr>
<td>INDO</td>
<td>13.6 ± 2.8</td>
<td>−7.2 ± 1.3</td>
<td>22,033 ± 1695</td>
<td>82.8 ± 12.6</td>
</tr>
<tr>
<td>INDO + LA-419</td>
<td>19.5 ± 3.9**</td>
<td>−3.7 ± 1.3**</td>
<td>18,429 ± 1370**</td>
<td>47.5 ± 9.3*</td>
</tr>
</tbody>
</table>

* P < 0.05, ** P < 0.01 vs. inflamed control (INDO) group.

TABLE 2
Effect of LA-419 on food consumption, body weight, blood leukocytes, and TNF during the chronic phase (day 15–18) of indomethacin-induced enteritis in rats
Data are expressed as means ± S.E.M. (n = 12).

<table>
<thead>
<tr>
<th>Group</th>
<th>Food Consumption</th>
<th>%</th>
<th>Leukocytes</th>
<th>TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.7 ± 1.8</td>
<td>30.7 ± 2.3</td>
<td>11,379 ± 1,590</td>
<td>&lt;4</td>
</tr>
<tr>
<td>LA-419</td>
<td>26.2 ± 1.4</td>
<td>28.7 ± 2.2</td>
<td>12,675 ± 1,921</td>
<td>&lt;4</td>
</tr>
<tr>
<td>INDO (active)</td>
<td>25.1 ± 1.9</td>
<td>12.2 ± 2.1</td>
<td>18,059 ± 829</td>
<td>56.1 ± 10.8</td>
</tr>
<tr>
<td>INDO (inactive)</td>
<td>14,204 ± 1,154</td>
<td>32.1 ± 8.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>INDO + LA-419 (preventive treatment)</td>
<td>25.3 ± 1.7</td>
<td>20.2 ± 2.3**</td>
<td>13,283 ± 1,089**</td>
<td>10.9 ± 9.7*</td>
</tr>
<tr>
<td>INDO + LA-419 (therapeutic treatment)</td>
<td>26.3 ± 1.7</td>
<td>12.7 ± 2.3</td>
<td>16,213 ± 1,236*</td>
<td>27.8 ± 10.6</td>
</tr>
</tbody>
</table>

** P < 0.01 vs. inflamed control (INDO) group; * P < 0.05; †† P < 0.01 vs. inflamed control (INDO) group (active phase).

characterized by the presence of micro-ulcers, inflammatory infiltration in the lamina propria, submucosa, and serosa, a shortening of crypts, and mucin depletion. At the end of the study (day 18), very few inflamed areas were observed and were characterized by the presence of an inflammatory infiltration in the submucosa, long abnormal crypts, and an increase in the number of goblet cells. Whereas no differences were found in the lesion score from inflamed control group (1.34 ± 0.91) and inflamed group receiving LA-419 as a therapeutic treatment (1.46 ± 0.77), pretreatment of the animals with LA-419 significantly reduced the severity of acute inflammatory reaction, the lesion score being 0.78 ± 0.52 (P < 0.01 versus inflamed control group) at the end of the study. LA-419 administration to noninflamed animals did not induce any histological change (Fig. 1).

** Myeloperoxidase Concentration

As shown in Fig. 2, in control animals, MPO was detected in ileal tissue homogenates at a low concentration (6.06 ± 1.85 ng/ml). By contrast, MPO concentration significantly increased in ileum in rats with induced enteritis selected during the active phase of inflammation (23.22 ± 9.54 ng/ml, P < 0.01). Preventive and therapeutic administration of LA-419 reduced MPO values (10.15 ± 3.9** at ASPET Journals on April 1, 2017 jpet.aspetjournals.org Downloaded from 7.2, respectively) compared to the inflamed control group. LA-419 in noninflamed animals did not induce any change in MPO concentration (4.13 ± 1.67 ng/ml).

** iNOS and nNOS mRNA Expression

As shown in Fig. 3, a low expression of iNOS mRNA was detected in the ileum of both controls (0.11 ± 0.04) and noninflamed animals receiving LA-419 (0.08 ± 0.03). As described previously (Porras et al., 2006b), animals with indo-

** Fig. 1. Representative photographs showing histological images of intestines obtained from control animal (A), LA-419 control (B), indomethacin animal selected during the active phase (C), indomethacin animal receiving LA-419 as preventive treatment (D), and indomethacin animal receiving LA-419 as a therapeutic treatment (E).

** Fig. 2. Bar diagram showing average MPO concentration in each group. Bars represent the mean ± S.E.M.; n = 6 rats/group. Prev, preventive treatment; ther, therapeutic treatment. ** P < 0.01; *** P < 0.001 versus control group. †† P < 0.01 between inflamed control group (active phase) and inflamed LA-419-pretreated group.
Regarding the constitutive nNOS isoform, a similar expression of the enzyme was observed in both controls (0.83 ± 0.10) and noninflamed animals receiving LA-419 (0.93 ± 0.17). As previously described, a reduction in the expression of nNOS was found in animals with indomethacin-induced enteritis, independently of the phase in which animals were selected (active phase: 0.46 ± 0.08, P < 0.01; inactive phase: 0.54 ± 0.11; P < 0.05). Whereas therapeutic treatment of inflamed animals with LA-419 partially restored nNOS expression (0.62 ± 0.09), pretreatment of the animals with LA-419 suppressed the reduction of nNOS expression, this value being similar to that observed in controls (0.89 ± 0.11).

**COX-2 and COX-1 mRNA Expression**

Similarly with iNOS expression, a low expression of COX-2 mRNA was detected in the ileum of both controls (0.10 ± 0.05) and noninflamed animals receiving LA-419 (0.09 ± 0.03). By contrast, an oscillation in the ileal COX-2 mRNA expression was observed in animals with indomethacin-induced inflammation. As shown in Fig. 4, whereas COX-2 mRNA expression significantly increased during the active phases of inflammation (0.36 ± 0.09, P < 0.05), a return to normal levels was observed during the inactive phases (0.19 ± 0.05). Whereas therapeutic treatment of inflamed animals with LA-419 partially reduced COX-2 overexpression (0.26 ± 0.06), pretreatment of the animals with LA-419 totally suppressed the enhancement of COX-2 expression to a value similar to that observed in healthy controls (0.15 ± 0.08). In addition, both preventive and therapeutic LA-419 treatments were effective in preventing the cyclical oscillation of COX-2. No differences between groups were observed regarding the expression of the constitutive COX isoform.

**Bacterial Translocation**

Results from aerobic bacteria isolated from the mesenteric lymph nodes in all groups are summarized in Table 3. As described previously (Porras et al., 2006a), a significant increase in positive mesenteric lymph nodes cultures was found in inflamed rats selected during the active phase of inflammation compared with the control group, with *Escherichia coli* and *Enterococcus sp.* being the species most frequently isolated. Administration of LA-419 to inflamed animals reduced the incidence of bacterial translocation. In the LA-419-pretreated group, bacterial translocation results were similar to those found in the control healthy group.

**Intestinal Motor Activity**

**Spontaneous Motor Activity.** As described previously (Porras et al., 2004), spontaneous motor activity in noninflamed control animals was characterized by isolated phasic contractions occurring at regular frequency. As shown in Fig. 5, this pattern was modified in inflamed rats. Whereas motor activity increased significantly during the inactive phases, a severe hypomotility was observed during the active phases of inflammation. Administration of LA-419 to inflamed animals in either preventive or therapeutic treatments reduced these motility disturbances, the spontaneous motility in treated animals similar in form and frequency of contractions to those of the noninflamed control group. LA-419 in noninflamed animals did not induce any motor change.

**Response to CCK.** In noninflamed control rats, CCK-8 infusion induced a contractile response at the duodenum

methacin-induced enteritis showed an oscillation of iNOS expression according to the phase in which animals were selected. Whereas, iNOS mRNA expression significantly increased during the active phase of inflammation (0.25 ± 0.08; P < 0.05), a return to normal levels was observed during the inactive phase (0.15 ± 0.04). In the inflamed groups receiving LA-419 as either preventive or therapeutic treatment, iNOS mRNA expression was similar to the control group (0.09 ± 0.03 and 0.11 ± 0.05, respectively). Moreover, LA-419 treatment was effective in preventing the characteristic oscillation of iNOS mRNA expression found in animals with induced inflammation.

**Toxicity.** Throughout the experimental period, no significant differences in leukocyte levels were selected in active and inactive phases, according to their blood cell composition. C, control animal; A, active phase; I, inactive phase; P, preventive treatment; T, therapeutic treatment. Bar diagram showing semiquantitative analysis by reverse transcription-PCR of iNOS and nNOS mRNA. Values are means ± S.E.M. of n = 4–6 in each group. *, P < 0.05; **, P < 0.01 versus control group; +, P < 0.05; +++, P < 0.001 between inflamed control group (active phase) and inflamed LA-419-treated group.

**Fig. 3.** A, representative photographs showing the expression of iNOS and nNOS mRNA in ileum. Rats with indomethacin-induced enteritis were selected in active and inactive phases, according to their blood leukocyte levels. C+, positive control; C, control animal; A, active phase; I, inactive phase; P, preventive treatment; T, therapeutic treatment. B, bar diagram showing semiquantitative analysis by reverse transcription-PCR of iNOS and nNOS mRNA. Values are means ± S.E.M. of n = 4–6 in each group. *, P < 0.05; **, P < 0.01 versus control group; +, P < 0.05; +++, P < 0.001 between inflamed control group (active phase) and inflamed LA-419-treated group.
AUC/min values increased from 5.14 ± 0.67 mm² to 59.81 ± 16.12 mm², \( P < 0.001 \). As shown in Fig. 6, the magnitude of CCK-induced contraction in inflamed control animals was higher during the inactive phase (76.25 ± 20.73 mm²) and lower during the active phase (34.12 ± 12.13 mm²) compared to the response observed in control animals. Both preventive and therapeutic LA-419 treatments restored CCK-induced contractile response in the duodenum to control values (46.51 ± 6.62 and 45.58 ± 6.43 mm², respectively). In control animals, CCK has an inhibitory effect in the jejunum similar to that observed in previous studies (data not shown) (Giralt and Vergara, 1999). This inhibitory response was not modified in inflamed rats or animals treated with LA-419.

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>E. coli</th>
<th>Enterococcus sp.</th>
<th>Lactobacillus sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1/6</td>
<td>0/6</td>
<td>1/6</td>
</tr>
<tr>
<td>LA-419</td>
<td>1/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>INDO (active)</td>
<td>4/6</td>
<td>4/6*</td>
<td>2/6</td>
</tr>
<tr>
<td>INDO (inactive)</td>
<td>1/6</td>
<td>2/6</td>
<td>1/6</td>
</tr>
<tr>
<td>INDO + LA-419 (preventive treatment)</td>
<td>2/6</td>
<td>1/6</td>
<td>0/6</td>
</tr>
<tr>
<td>INDO + LA-419 (therapeutic treatment)</td>
<td>3/6</td>
<td>3/6*</td>
<td>1/6</td>
</tr>
</tbody>
</table>

* \( P < 0.05 \) compared with control group.

Table 3

Presence of bacterial translocation for a specific microorganism in control group and rats with indomethacin-induced enteritis: effect of LA-419

Results are expressed as positive cases/number total of rats, \( n = 6 \) in each group.

Fig. 4. A, representative photographs showing the expression of COX-2 and COX-1 mRNA in ileum. Rats with indomethacin-induced enteritis were selected in active and inactive phases according to their blood leukocyte levels. C+, positive control; C, control animal; A, active phase; I, inactive phase. B, bar diagrams showing semiquantitative analysis by reverse transcription-PCR of COX-2 and COX-1 mRNA. Values are means ± S.E.M. of \( n = 4–6 \) in each group. \( *, P < 0.05 \) versus control group; \( **, P < 0.01 \) between inflamed control group (active phase) and inflamed LA-419 group.

Fig. 5. A, representative mechanical recordings of the spontaneous motor activity in the duodenum from one animal of each experimental group. Prev, preventive treatment; ther, therapeutic treatment. B, total number of spontaneous contractions recorded at duodenum in all experimental groups. Similar results were found in the jejunum. Values are means ± S.E.M. of \( n = 5–6 \) in each group. \( *, P < 0.05 \); \( **, P < 0.01 \) versus control group. \( ***, P < 0.001 \) versus control group; \( **, P < 0.01 \) between inflamed control group (active phase) and inflamed LA-419 group.
This study demonstrates that, in a rat model of indomethacin-induced enteritis, NO-donor LA-419 exerts a beneficial effect on intestinal inflammation. When administered as a preventive treatment, LA-419 reduces the severity of the acute phase of inflammation and prevents relapses occurring during the chronic stage. Administration of LA-419 after inflammation was induced, although less effective, also im-
proved inflammatory parameters. These effects of LA-419 are derived from the modulation exerted by the NO-releasing compound on the expression of both iNOS and nNOS isoenzymes, iNOS and nNOS.

In our experimental model, active and inactive phases were associated with a dysregulated expression of both iNOS and nNOS (Porras et al., 2006b). As constitutively synthesized iNOS is involved in the regulation of multiple physiological processes (Alican and Kubes, 1996; Jourdeheuil et al., 1999), decreased NO synthesis derived from down-regulated nNOS could have numerous deleterious consequences for the intestine, contributing to the development and chronicity of intestinal inflammation. Moreover, high NO levels produced by up-regulated iNOS during the active phase cause intestinal hypomotility, which results in luminal bacterial overgrowth and translocation of bacteria across the bowel wall, factors that could precipitate or contribute to relapse of inflammation (Nieuwenhuijs et al., 1998; Gunnarsdottir et al., 2003). In addition, excess of NO may also be involved in the chronicity of intestinal damage by a number of additional mechanisms, including disruption of the epithelial barrier (Kubes, 2000; Virag et al., 2003).

Our hypothesis was that maintenance of physiological levels of NO, produced by a treatment with exogenous NO, could prevent the deleterious effects derived from the nNOS down-regulation at the same time that avoiding overproduction of NO induced by the up-regulation of iNOS occurs during the active phase. When given before induction of intestinal inflammation, LA-419 reduced the enhancement of inflammatory blood parameters during the acute stage. At the end of the study (day 18), a decrease of inflammatory parameters paralleled by a concomitant reduction in small intestinal macroscopic and histological scores was also observed. These effects were associated with a normal expression of iNOS and nNOS. On the other hand, although the treatment with LA-419 was less effective after induction of intestinal inflammation, three main features should be considered about the effects of this compound. 1) LA-419 blocks the relapse of the active phase, 2) inflammatory parameters tended to return to the normal values, and 3) a normalization in the expression of iNOS and nNOS was observed. Further studies are needed to evaluate whether a more prolonged treatment with LA-419 may result in a complete resolution of the inflammatory process.

It has been described that iNOS knockout animals (Beck et al., 2007) are more resistant to dextran sulfate sodium-induced inflammation, suggesting that blockade of iNOS up-regulation is crucial to reduce the severity of the process. This blockade of iNOS was achieved by administration of LA-419, and several potential mechanisms may be implicated in this effect. It is known that the expression of iNOS is regulated predominantly at the transcriptional level through mechanisms dependent on nuclear factor-κB (NF-κB) (Taylor and Geller, 2000) and that activation of NF-κB is inhibited by NO derived from nNOS (Peng et al., 1995). Therefore, nNOS down-regulation observed in our study could be responsible for the up-regulation of iNOS that occurs during the active phase. Hence, blockade of nNOS down-regulation by LA-419 can avoid NF-κB activation, resulting in the prevention of the iNOS up-regulation that occurs during the active phase of the inflammation. This effect of LA419 could be attributed to NO levels produced by this compound, as supported by recent studies showing an inhibition of NF-κB activation due to exogenously applied NO (Dijkstra et al., 2002). Treatments with NO-donors can be effective by acting at initial level of the inflammatory cascade. Regarding the nNOS down-regulation observed in inflamed nontreated animals, it has been described that overproduction of NO resulting from iNOS up-regulation is responsible for the nNOS down-regulation at a neuronal level (Mizuta et al., 2000). Therefore, blockade of iNOS up-regulation induced by LA-419 could prevent nNOS down-regulation observed during the inflammatory state. This normalization in the iNOS/nNOS balance could represent a fundamental factor in the improvement of the inflammatory process.

One important beneficial effect of the maintenance of iNOS/nNOS balance can be observed at the gastrointestinal motility level. Administration of LA-419 before the induction of inflammation resulted in the recovery of the normal intestinal motor parameters, including the response to L-NNa, which reflects the state of the inhibitory innervation pathways. Recovery of intestinal motility could lead to a decrease in bacterial overgrowth and, hence, contribute to the absence of bacterial translocation observed after LA-419 administration. Although a trend to normalization of some motility parameters was also observed when LA-419 was administered after the induction of intestinal inflammation, the response after L-NNa was still altered, indicating that intestinal motility had not fully recovered. This fact might be related to the translocation of bacteria occurring in these animals.

Our results about the beneficial effects of LA-419 in the treatment of intestinal inflammation are in agreement with Tanaka et al. (2001), who demonstrated that treatment with the NO-donor NOR-3 prevented the development of intestinal lesions in the acute phase of indomethacin-induced enteritis in the rat, an effect that was attributed to the inhibition of enterobacterial translocation. Moreover, several studies demonstrate a significant reduction on side effects caused by administration of NO-releasing nonsteroidal anti-inflammatory drugs compared to ordinary nonsteroidal anti-inflammatory drugs (Bandarage and Janero, 2001; Tomisato et al., 2005), a fact that seems to be related to a decrease of iNOS activity induced by these compounds (Rao et al., 2006). These data support our hypothesis that amelioration of inflammation induced by LA-419 may be related to the maintenance of nNOS/iNOS balance.

In conclusion, our results demonstrate that the LA-419 treatment exerts a protective effect during the acute phase of indomethacin-induced enteritis in the rat. Moreover, LA-419 can also be useful as a therapeutic agent, although further studies with a more prolonged duration of the treatment are needed. LA-419-protective effect is associated to the normalization of inflammatory parameters, the prevention of intestinal dysmotility, and the suppression of bacterial translocation. Therefore, LA-419 might be a novel therapeutic approach to prevent acute inflammatory relapses in patients with IBD.

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