Cyclical upregulated iNOS and long-term downregulated nNOS are the bases for relapse and quiescent phases in a rat model of IBD

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Porras, M., M. T. Martín, R. Torres, and P. Vergara. Cyclical upregulated iNOS and long-term downregulated nNOS are the bases for relapse and quiescent phases in a rat model of IBD. Am J Physiol Gastrointest Liver Physiol 290: G423–G430, 2006. First published October 20, 2005; doi:10.1152/ajpgi.00323.2005.—We previously reported that indomethacin induces a chronic intestinal inflammation in the rat where the cyclical characteristic phases of Crohn’s disease are manifested with a few days’ interval and lasting for several months: active phase (high inflammation, hypomotility, bacterial translocation) and reactive phase (low inflammation, hypermotility, no bacterial translocation). In this study, we investigated the possible role of both constitutive and inducible isoforms of nitric oxide (NO) synthase (NOS) and cyclooxygenase (COX) in the cyclicity of active and reactive phases in rats with chronic intestinal inflammation. Rats selected at either active or reactive phases and from 2 to 60 days after indomethacin treatment were used. mRNA expression of both constitutive and inducible NOS and COX isoforms in each phase was evaluated by RT-PCR and cellular enzyme localization by immunohistochemistry. The effects of different COX and NOS inhibitors on the intestinal motor activity were tested. mRNA expression of COX-1 was not modified by inflammation, whereas mRNA expression of neuronal NOS was reduced in all indomethacin-treated rats. In contrast, NOS and COX inducible forms showed a cyclical oscillation. mRNA expression and protein of both inOS and COX-2 increased only during active phases. The intestinal hypomotility associated with active phases was turned into hypermotility after the administration of selective iNOS inhibitors. Sustained downregulation of constitutive NOS caused hypermotility, possibly as a defense mechanism. However, this reaction was masked during the active phases due to the inhibitory effects of NO resulting from the increased levels of the inducible NOS isoform.

nitric oxide; prostaglandins; intestinal inflammation; dysmotility; inducible nitric oxide synthase; neuronal nitric oxide synthase

Inflammatory bowel disease (IBD) includes two chronic pathologies characterized by alternation of active and quiescent phases of inflammation: ulcerative colitis and Crohn’s disease (9). Although its pathogenesis has not been well established, it has been suggested that some clinical manifestations frequently observed in IBD patients, such as abdominal pain, nausea, vomiting, ileus, or diarrhea, could be attributed to the deranged gastrointestinal motility associated with inflammation (5). Furthermore, an overproduction of nitric oxide (NO) and prostaglandins has been detected in inflamed intestinal samples in both human and experimental IBD (1, 7).

In a healthy state, constitutive neuronal NO synthase (nNOS) produces basal levels of NO to maintain the physiological tonic inhibition of the intestine (6). Moreover, NO derived from nNOS also plays a role in the control of small intestinal motor parameters, i.e., inducing the conversion of fasting motility into a postprandial pattern (23). Prostaglandins produced by constitutive COX (COX-1) also modulate some gastrointestinal functions under physiological conditions, inducing contractibility of smooth muscles and neurotransmitter release (10).

During inflammation, inducible isoforms of NOS (iNOS) and COX (COX-2) are produced in response to different stimuli, such as LPS and proinflammatory cytokines (8, 13). Although several studies (14, 15, 26) have focused on the relationship between overproduction of NO and prostaglandins derived from iNOS and COX-2 and the disturbed motor activity related to intestinal inflammation, their specific pathophysiological effect on gut motility is still controversial.

By modifying a previously reported protocol (24), we have obtained a new chronic model of IBD induced by indomethacin and characterized by spontaneous cyclical alternation of active and reactive phases of inflammation, which are correlated with long-lasting motor disturbances (21). In this model, active phases of inflammation were related to high blood leukocyte and TNF concentration as well as a reduced motor activity, bacterial overgrowth, and bacterial translocation into the intestinal wall. In contrast, reactive phases were related to a reduction of inflammatory parameters, hypermotility, and normal bacterial load. This reactive phase is equivalent to the quiescent periods in IBD disease.

Because of the relevant role of NO during inflammatory states, we hypothesized that hypomotility associated with the active inflammatory phases of our model is related to an overproduction of NO derived from increased levels of iNOS. However, because coexpression of iNOS and COX-2 has been reported in intestinal inflammation, a putative role of prostaglandins in the disturbed motor activity cannot be dismissed. Thus the aims of the present study were 1) to evaluate the mRNA expression of the constitutive and inducible isoforms of both NOS and COX by RT-PCR in both active and reactive phases of inflammation; 2) to confirm and localize iNOS and COX-2 protein by immunohistochemistry; and 3) to determine the specific contribution of these enzymes on the deranged motor activity by testing the effects of different NOS and COX enzyme inhibitors on the intestinal motility of anesthetized rats.

Materials and Methods

Animals. Male Sprague-Dawley rats (Charles River, Lyon, France), 8–10 wk old and weighing 300–350 g, were used. They were kept

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under conventional conditions in an environmentally controlled room (20–21°C, 60% humidity, 12:12-h light-dark cycle) with tap water and standard laboratory rat chow ad libitum. All experimental protocols were approved by the Ethical Committee of the Universitat Autònoma de Barcelona.

Experimental model. Intestinal inflammation was induced by administration of two subcutaneous injections of indomethacin (7.5 mg/kg in a 0.3-ml volume) 48 h apart, as previously described (21). This model shows a spontaneous alternation of active and reactive phases of inflammation, where active phases are characterized by a generalized hypomotility, bacterial overgrowth, and increase of blood leukocytes and TNF levels, whereas reactive phases are associated to hypermotility, recovery of the normal intestinal flora load, and reduction of blood leukocytes and TNF levels. Blood leukocyte monitoring allowed the selection of animals during active (high blood leukocytes) and reactive (low blood leukocytes) phases of inflammation.

Experimental groups. Two groups of animals were used: 1) the indomethacin-treated group (n = 44) and 2) the control group (n = 12), which received saline. Studies were performed 2 days after indomethacin administration (acute stage) as well as 15 ± 3, 30 ± 3, and 60 ± 3 days after treatment (chronic stage), with the animals selected in active and reactive phases according to their blood leukocyte values.

RT-PCR studies. Ten-centimeter samples from the distal jejunum and the midileum of control and indomethacin-treated rats were taken immediately after the animals were killed by CO₂ inhalation. As a positive control of iNOS and COX-2 mRNA expression, four additional rats exposed to LPS (15 mg/kg ip) were used (16). Intestinal segments were divided into four equal parts, and each sample was frozen in liquid nitrogen and stored immediately at −80°C until use.

RNA extraction. Total RNA was extracted from samples using RNAwiz (Ambion, Madison, WI) according to the manufacturer’s instructions and treated with DNA-free (Ambion) for 30 min at 37°C to remove any genomic DNA contamination.

RT-PCR. First-strand cDNA was synthesized from 5 µg total RNA in a reaction mixture of 50 µl containing 0.5 µg of oligo18(dT) primer (Ambion), 2 mM dNTP (Ecogen, Barcelona, Spain), and 10 units Moloney murine leukemia virus (Ambion). The resultant cDNA was amplified in a total volume of 50 µl with 1 units of tagDNA, 1 mM dNTP mixture, and 0.5 µM primers (Prolog). The sequences of sense and antisense primers for rat iNOS, nNOS, COX-2, COX-1, and GAPDH (a constitutively expressed gene used as a control of the efficiency of cDNA synthesis) are listed in Table 1. The PCR amplification protocol was as follows: 35 cycles with 1 min of denaturation at 95°C; 1 min of annealing at 56°C (COX-2), 53°C (nNOS), or 50°C (COX-1, iNOS, and GAPDH); and 1 min of extension at 72°C on a thermal cycler. Amplified products were electrophoresed on 1.5% agarose gel in TAE buffer, stained with ethidium bromide, photographed under ultraviolet light, and quantified using image-analyzing software (Quantity-One, Bio-Rad Laboratories). For semiquantification, the ratio of the optical density of each PCR product and GAPDH was determined.

Immunohistochemistry of iNOS and COX-2. Immunohistochemistry of iNOS and COX-2 was performed in tissue sections (5 µm) of both the jejunal and ileal prepared on adhesive-coated slides, deparaffinized, and rehydrated. Sections were treated for antigen retrieval by boiling in 10 mM citrate buffer (pH 6) for 15 min. After being washed with PBS, sections were incubated in 5% hydrogen peroxide to block endogenous peroxidase activity and washed again with PBS. Non-specific binding was blocked with a 20% goat (for detecting iNOS) or rabbit (for detecting COX-2) serum solution in PBS for 1 h at room temperature. Sections were incubated overnight at 4°C with anti-iNOS antibody (1:100; Neo-markers, Fremont, CA) or anti-COX-2 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). In additional control sections, the primary antibody was omitted. A biotinylated goat anti-rabbit (for detecting iNOS) or rabbit anti-goat (for detecting COX-2) IgG (Santa Cruz Biotechnology) was used as a secondary antibody diluted in PBS at 1:200. Sections were then treated with an avidin-biotin horseradish peroxidase kit (Vector Laboratories), and reaction products were detected using a diaminobenzidine substrate kit for peroxidase (Vector Laboratories).

Motility studies. Motor activity was evaluated in the indomethacin group 30 ± 3 days after treatment in both active and reactive phases of inflammation as well as in the control group. Surgical procedure was performed as previously described (21). Briefly, animals fasted for 6 h were anesthetized by halothane inhalation. The right jugular vein was cannulated to maintain level III of anesthesia by administration of thiopental sodium as required. A laparotomy was performed to suture three strain gauges (3 × 5 mm, Hugo Sachs Elektronik, Germany) to the wall of the duodenum (at 2 cm from the pylorus), proximal jejunum (at 2 cm from Treitz’s ligament), and ileum (at 10 cm from cecum) to record circular muscle activity. Strain gauges were connected to high-gain amplifiers (MT8P; Lectromed, UK), and signals were sent to a recording unit connected to a computer (PowerLab/800; ADInstruments).

After an equilibration period of 10 min, spontaneous motor activity was recorded for 1 h. To evaluate the effects of NOS inhibitors, motility was recorded for 1 h after intravenous administration of L-Nω-(1-iminoethyl)-lysine (L-NIL), a selective iNOS inhibitor (4 mg/kg). Immediately after, Nω-nitro-L-arginine (L-NNA; 10–3 mol/L), a nonselective NOS inhibitor, was injected, and motor activity was recorded for 1 h more. To test the effect of COX-2 inhibition, a separate set of animals was used and motility was recorded for 1 h after the administration of SC-58125 (10 mg/kg sc), a selective

Table 1. Sequences of sense and antisense primers for rat COX-1, COX-2, nNOS, iNOS, and GAPDH

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sequence</th>
<th>PCR Product, bp</th>
<th>cDNA Position</th>
<th>NCBI Refseq</th>
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<tbody>
<tr>
<td>COX-1</td>
<td>Sense 5'-CTCTCAAGACGAAAGCCTG-3'</td>
<td>402</td>
<td>1765–1782</td>
<td>S67721</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-CAGCCTGGCTCTGTGATAT-3'</td>
<td>2149–2167</td>
<td>S67722</td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>Sense 5'-CAAACGAGGACTGCTCAA-3'</td>
<td>483</td>
<td>2996–3013</td>
<td>NM-052799</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-CCCCTTTACCTGTAGATT-3'</td>
<td>3462–3479</td>
<td>NM-012611</td>
<td></td>
</tr>
<tr>
<td>nNOS</td>
<td>Sense 5'-CTCTCTTGCCACTAATG-3'</td>
<td>335</td>
<td>3541–3558</td>
<td>NM-012611</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-GACTACATGGTACGCTT-3'</td>
<td>3858–3875</td>
<td>NM-012611</td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>Sense 5'-CCGATATTCTTCTTGACATA-3'</td>
<td>272</td>
<td>493–511</td>
<td>NM-012611</td>
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<tr>
<td></td>
<td>Antisense 5'-GCTTGCTGCATATGTG-3'</td>
<td>848–865</td>
<td>NM-012611</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense 5'-CAGGCCCCTCCGCTATGCG-3'</td>
<td>140</td>
<td>1208–1227</td>
<td>NM-017008</td>
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<tr>
<td></td>
<td>Antisense 5'-ATGACCCCTCTCCGACAGTG-3'</td>
<td>1348–1367</td>
<td>NM-017008</td>
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</tbody>
</table>

COX, cyclooxygenase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase.
COX-2 inhibitor. Motor responses were quantified by measuring the area under the curve per minute and are expressed in square millimeters.

Drugs and solutions. Lipopolysaccharide 0111:B4 (Escherichia coli), L-NNa, and L-NIL from Sigma (St. Louis, MO) were dissolved in saline solution. Indomethacin (Sigma) was dissolved in absolute ethanol (50 mg/ml) and diluted in a 0.1 M NaHCO₃ solution. 5-(4-Fluorophenyl)-1-[4-(methylsulfonyl)-phenyl]-3-(trifluoromethyl)-1H-pyrazole (SC-58125) from Cayman Chemical (Ann Arbor, MI) was dissolved in DMSO (20 mg/ml).

Data analysis and statistics. Data are expressed as means ± SE. Differences in mRNA expression between groups were compared using one-way ANOVA and Bonferroni’s post hoc analysis. In motility studies, a paired t-test was used to compare the effects of COX and NOS inhibitors vs. spontaneous motor activity. Results were considered to be statistically significant when \( P < 0.05 \).

RESULTS

iNOS and nNOS mRNA expression. After 35 cycles of PCR using specific primers for iNOS, nNOS, and GAPDH as an internal standard, single bands for each cDNA at the expected size were observed. The ratios of iNOS and nNOS to GAPDH for each group are shown in Fig. 1.

In control animals, a low expression of iNOS mRNA was detected in the ileum. Indomethacin rats selected 2 days after treatment showed a high increase in both jejunal and ileal iNOS mRNA expression, whereas in the chronic stage, an oscillation of iNOS expression was found according to the phase in which animals were selected. As shown, iNOS mRNA expression significantly increased during the active phases of
inflammation, whereas a return to normal levels was observed during the reactive phases.

Regarding the constitutive NOS isoform, a similar expression of the enzyme was observed in control animals in both jejunal and ileal segments. By contrast, a reduction in the expression of nNOS was found in animals 2 days after indomethacin administration. This decrease of nNOS mRNA expression was maintained until 30 days after indomethacin treatment, independently of the phase in which animals were selected.

COX-2 and COX-1 mRNA expression. Similarly to iNOS expression, a high increase in both the jejunal and ileal COX-2 mRNA expression was observed in indomethacin-treated animals 2 days after administration, whereas differences in the COX-2 mRNA expression were observed in the chronic stage according to the phase in which animals were selected (Fig. 2). Whereas COX-2 mRNA expression was significantly increased during the active phases of inflammation, a return to normal levels was observed during the reactive phases.

Regarding the constitutive COX isoform, a similar expression was found in control and indomethacin-treated rats, independently of the phase in which they were selected.

iNOS and COX-2 immunohistochemistry. In control animals, no immunostaining of any inducible isoform was observed in jejunal tissues, whereas only weak iNOS and COX-2 immunoreactivity was detectable in the cytoplasm of enterocytes located in the apical side of ileal villi, as shown in Fig. 3. In animals selected 2 days after indomethacin treatment, marked COX-2 and iNOS immunoreactivity was evident throughout the gut wall in both intestinal segments but was particularly noticeable in the cytoplasm of epithelial cells. In the chronic stage, a similar distribution of both proteins was observed in samples from animals selected at active phases of inflammation. In these animals, positive staining was intense in the

Fig. 2. A: representative photographs showing the expression of cyclooxygenase (COX)-2 and COX-1 mRNA in J and I from control and indomethacin-treated rats selected in acute (day 2) and chronic inflammatory stage (days 15, 30, and 60 after treatment). Rats with chronic inflammation were selected in active and reactive phases, according to their blood leukocyte levels. B: bar diagrams showing semiquantitative analysis by RT-PCR of COX-2 and COX-1 mRNA in J and I of control and indomethacin-treated rats. Values are means ± SE of n = 4–6 in each group. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the control group.
mucosa, including the cytoplasm of enterocytes and cells in the lamina propria. Additional staining in cells located in the submucosa, muscle layers, and serosa was also observed. By contrast, during the reactive phases, only a weak immunostaining was observed in the cytoplasm of some enterocytes located in the apical side of villi, similar to that found in control animals.

**Motility studies.** As previously described (21), spontaneous motor activity in control animals was characterized by isolated phasic contractions occurring at regular frequency. All indomethacin-treated rats selected at different phases of inflammation showed an alteration of this typical motor pattern. Whereas motor activity increased significantly during the reactive phases, a severe hypomotility was observed during the active phases of inflammation. As shown in Fig. 4, administration of L-NIL (a selective iNOS inhibitor) failed to modify motor activity in control and indomethacin-treated rats selected during the reactive phase, whereas during the active phase of inflammation, L-NIL induced a significant increase of motor activity, with this induced motility similar to that observed during the reactive phases. Administration of L-NNA (a non-selective NOS inhibitor) resulted in an increase in the motor activity in all groups studied. However, the effect of L-NNA was higher in the control group than in treated animals.

With respect to the results obtained after COX-2 activity inhibition, administration of SC-58125 did not change the motor activity in neither control nor indomethacin-treated rats selected during the reactive phase, whereas a slight increase of
motor activity was observed in rats selected during the active phase, with this induced motility similar to that observed in control animals (Fig. 5).

**DISCUSSION**

Using an experimental model of intestinal inflammation induced by indomethacin, we previously described oscillations of active and reactive phases of inflammation that are similar to the active and quiescence episodes observed in IBD patients. During the active phases, an increase of inflammatory parameters associated with hypomotility and bacterial translocation was observed in indomethacin-treated rats, whereas the reactive phases were characterized by a decrease of these inflammatory parameters, hypermotility, and normal bacterial load (21).

In the present study, the results derived from using this model demonstrate that NO derived from the upregulated iNOS is responsible for the hypomotility associated with the active phases of the disease. Furthermore, this study shows a downregulation of nNOS that is maintained through the chronic inflammatory state and causes a decrease in the inhibitory tone of the intestine. Both isoenzymes seem to be responsible for the cyclical hypo-hypermotility pattern.

The upregulation of both iNOS and COX-2 mRNA observed during the active phases suggests that both pathways are coexpressed as previously reported using other models of inflammation (20, 28). However, in contrast to previous reports relating the coexpression of both enzymes to the disturbed motor activity observed in the course of intestinal inflammation (14, 15), our results do not corroborate an equal participation of both coenzymes in the hypo-hypermotility cycle. Our motility results clearly demonstrate that hypomotility is mainly a consequence of an overproduction of NO. In contrast, COX-2 inhibitor at the dose used did not substantially modify motility parameters. This dose has been reported to be effective in other inflammatory studies (25, 29). In consequence, and in contrast to that suggested in other studies (14, 26), prostaglandins induced by COX-2 seem to play a nonsignificant role in either the hypomotility or during hypermotility. This discrepancy may indicate that the mechanisms controlling motor activity are more susceptible to prostaglandins induced by COX-2 in the course of acute inflammation than to prostaglandins produced in a more chronic inflammatory state. Intestinal motor activity is an important regulator of luminal bacterial load (11, 19), and, in consequence, hypomotility induced by NO overproduction during the active phases of inflammation could be the cause of the bacterial overgrowth observed in our previous study with indomethacin-treated rats (21) as well as in IBD patients (12).

Another interesting finding of the present study is that, in contrast to the unchanged COX-1 expression, a long-lasting
decrease in the mRNA expression of nNOS was observed during both phases of inflammation. This result is in agreement with previous reports describing impaired synthesis of NO caused by reduction of immune-reactive nNOS cells in experimental models of colitis (4, 18). However, to our knowledge, this is the first study demonstrating downregulation of nNOS expression by RT-PCR in a model of enteritis. We observed this downregulation 2 days after induction of inflammation, suggesting that it is an early event, probably occurring as soon as the inflammatory process is initiated.

In comparison to NO derived from iNOS, the role of NO released from nNOS has been poorly studied. However, NO derived from nNOS is responsible for the physiological inhibitory tone of the intestine (3, 6). In consequence, the downregulation of this enzyme during intestinal inflammation causes hypermotility and could be part of the integrative defensive mechanisms existing in the gut to expel a noxious cause. A defensive reaction of the gut consisting on hypermotility is observed in inflammatory experimental models of nematode infection (27), facilitating the parasite expulsion and spontaneous cure. Our results show that nNOS downregulation causes hypermotility in a similar way as in those models of inflammation with spontaneous cure. Moreover, this reaction seems to be present for as long as the inflammation persists, but it is cyclically masked by the overexpression of iNOS causing hypomotility.

The suppression of nNOS gene expression has been attributed to large amounts of cytokines released during the acute phase of inflammation (2). In addition, it has been suggested that NO derived from nNOS activity keeps iNOS suppressed under normal conditions, whereas in the course of intestinal inflammation, downregulation of nNOS is a necessary condition to facilitate the expression of iNOS and the release of large amounts of NO (22). However, our results show that iNOS regulation is more complex. nNOS downregulation could induce iNOS overexpression, but our results also show that iNOS has a cyclical expression, whereas nNOS expression remains continuously downregulated. Another study (17) suggests that the large amounts of NO as the result of the overexpression of iNOS constitute a negative feedback mechanism of iNOS expression. Our results could be in agreement with this hypothesis: downregulation of nNOS induces upregulation of iNOS; however, iNOS expression could be downregulated when NO production is high.

Previous studies from our laboratory have demonstrated sustained impairment of epithelial barrier function in animals with induced inflammation (unpublished data). Our hypothesis is that whereas antigen load is maintained at lower level, due to
normal or enhanced motility (as in reactive phases), there is no inflammatory response despite the increased intestinal permeability. However, when motility decreases and bacterial load rises, a higher proportion of antigens can interact with the mucosal surface and traverse through the dilated tight junctions, resulting in the activation of the immune system and the upregulation of iNOS (and COX-2).

In conclusion, regulation of both iNOS and nNOS plays a fundamental role in the course of intestinal inflammation. Whereas nNOS is long term downregulated, iNOS expression only increases cyclically during the active phases of inflammation. Downregulation of nNOS could act as a defensive mechanism by reducing intestinal tonic inhibition and, hence, by allowing the increase of gastrointestinal motility in an attempt to eliminate luminal noxious agents. However, as in IBD, this reaction seems unable to stop the cyclical relapse into another active phase, whereas upregulation of iNOS causes overproduction of NO-inducing intestinal hypomotility and then bacterial overgrowth.

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