Differential Role of Cyclooxygenase 1 and 2 Isoforms in the Modulation of Colonic Neuromuscular Function in Experimental Inflammation

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ABSTRACT

This study examines the role played by cyclooxygenase (COX) isoforms (COX-1 and -2) in the regulation of colonic neuromuscular function in normal rats and after induction of colitis by 2,4-dinitrobenzenesulfonic acid (DNBS). The expression of COX-1 and COX-2 in the colonic neuromuscular layer was assessed by reverse transcription-polymerase chain reaction and immunohistochemistry. The effects of COX inhibitors on in vitro motility were evaluated by studying electrically induced and carbachol-induced contractions of the longitudinal muscle. Both COX isoforms were constitutively expressed in normal colon; COX-2 was up-regulated in the presence of colitis. In normal and inflamed colon, both COX isoforms were mainly localized in neurons of myenteric ganglia. In the normal colon, indomethacin (COX-1/COX-2 inhibitor), SC-560 [5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole] (COX-1 inhibitor), or DFU [5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)-furanone] (COX-2 inhibitor) enhanced atropine-sensitive electrically evoked contractions. The most prominent effects were observed with indomethacin or SC-560 plus DFU. In the inflamed colon, SC-560 lost its effect, whereas indomethacin and DFU maintained their enhancing actions. These results were more evident after blockade of noncholinergic pathways. In rats with colitis, in vivo treatment with superoxide dismutase or S-methylisothiourea (inhibitor of inducible nitric-oxide synthase) restored the enhancing motor effect of SC-560. COX inhibitors had no effect on carbachol-induced contractions in normal or DNBS-treated rats. In conclusion, in the normal colon, both COX isoforms act at the neuronal level to modulate the contractile activity driven by excitatory cholinergic pathways. In the presence of inflammation, COX-1 activity is hampered by oxidative stress, and COX-2 seems to play a predominant role in maintaining an inhibitory control of colonic neuromuscular function.

Since the discovery of two cyclooxygenase (COX) isoforms (COX-1 and COX-2), efforts have been made to examine the distribution and functional roles played by these enzymes in the gastrointestinal tract. The mucosal layer of normal gut expresses high levels of COX-1 and low levels of COX-2 (Wallace and Devchand, 2005; Fornai et al., 2005b), whereas in the presence of ulcer, inflammatory, or neoplastic diseases, COX-2 expression can be up-regulated. In these settings, COX isoforms may play differential pathophysiological roles, such as the involvement of COX-1 and COX-2 in the development of gastrointestinal damage induced by nonsteroidal anti-inflammatory drugs (Tanaka et al., 2002), and the carcinogenesis and tumor cell proliferation promoted by COX-2-derived prostanooids (Subbaramiah and Dannenberg, 2003). In contrast, the pattern of COX isoform expression in gut neuromuscular layers remains to be clarified. Previous reports described a significant COX-2 expression in colonic myenteric plexus and myocytes of various species, including humans, under normal conditions (Porcher et al., 2004; For...
nai et al., 2005a), whereas other authors reported a scarce or absent expression (Roberts et al., 2001; Schwarz et al., 2001).

Intestinal inflammation is associated with alterations of gut motility and may contribute to the development of digestive symptoms (Collins, 1996). The mechanisms underlying dysmotility are still uncertain, but altered functions of myenteric nerves and smooth muscle have been observed (Sharkey and Kroese, 2001). Previous studies reported that COX pathways are involved in the modulation of normal gastrointestinal motility (Porcher et al., 2002, 2004; Fornai et al., 2005a), and that in the presence of intestinal inflammatory reactions, COX-2 might contribute to the pathophysiology of related motor alterations (Schwarz et al., 2001; Linden et al., 2004). For instance, experimental ileus in rats, evoked by intestinal manipulation, was associated with enteric COX-2 induction, and treatments with selective COX-2 inhibitors significantly improved the intestinal contractile activity, both in vivo and in vitro (Schwarz et al., 2001). Moreover, experimental colitis induced by trinitrobenzenesulfonic acid in guinea pigs is characterized by increased COX-2 expression in the colonic wall, and the products of this enzyme seem to be responsible for an enhanced excitability of myenteric AH neurons. In this model, the inhibition of COX-2, but not COX-1, restored the normal electrical properties of AH neurons, whereas the application of prostaglandin E2 to inflamed colonic preparations decreased the afterhyperpolarization of AH neurons and slowed their accommodation rate (Manning et al., 2002; Linden et al., 2004). However, there is still uncertainty on the role of COX isoforms in motor alterations associated with chronic intestinal inflammation and on the hypothesis that COX-derived mediators may regulate differently gut motility under physiological or pathological conditions (Costa, 2004).

The present study was designed to compare the role of COX isoforms in the control of neuromuscular functions in normal rats and after the induction of colitis. For this purpose, we examined the expression and localization of COX-1 and COX-2 in the colonic neuromuscular layer and the effects of COX blockade on in vitro colonic motor activity, using selective and nonselective COX-inhibitors.

Materials and Methods

Animals. Albino male Sprague-Dawley rats (200–250 g body weight) were used throughout the study. They were housed in temperature-controlled rooms in a 12-h light/dark cycle at 22–24°C and 50 to 60% humidity. Their care and handling were in accordance with the provision of the European Union Council Directive 86/609, recognized, and adopted by the Italian Government.

Induction and Assessment of Colitis. Colitis was induced in accordance with the method described previously by Barbara et al. (2000). In brief, during anesthesia with diethyl ether, 30 mg of 2,4-dinitrobenzenesulfonic acid (DNBS) in 0.25 ml of 50% ethanol was administered intrarectally via a polyethylene PE-40 catheter inserted 8 cm proximal to the anus. Control rats received 0.25 ml of saline. Animals underwent subsequent experimental procedures 6 days after DNBS administration to allow a full development of histologically evident colonic inflammation. At that time, the animals were euthanized, and the severity of intestinal inflammation was evaluated macroscopically and histologically in accordance with the criteria previously reported by Wallace and Keenan (1990), as modified by Barbara et al. (2000). The macroscopic criteria were based on the following: presence of adhesions between colon and other intra-abdominal organs, consistency of colonic fecal material (indirect marker of diarrhea), thickening of colonic wall, presence and extension of hyperemia, and macroscopic mucosal damage (assessed with the aid of a ruler). Microscopic criteria were assessed by light microscopy on hematoxylin- and eosin-stained sections obtained from whole-gut specimens, taken from a region of inflamed colon immediately adjacent to the gross macroscopic damage and fixed in cold 4% neutral formalin diluted in phosphate-buffered saline (PBS). Histological criteria included: degree of mucosal architecture changes, cellular infiltration, external muscle thickening, presence of crypt abscess, and goblet cell depletion. All parameters of macroscopic and histological damage were recorded and scored for each rat by two observers blinded to the treatment.

Reverse Transcription-Polymerase Chain Reaction. Expression of mRNA coding for COX isoforms was assessed by reverse transcription (RT)-polymerase chain reaction (PCR). The analysis was performed on colonic specimens excised as reported above, subjected to mucosa and submucosa removal, snap-frozen in liquid nitrogen, and stored at −80°C. At the time of extraction, tissue samples were disrupted with cold glass pestles, and total RNA was isolated by TRIzol (Life Technologies, Carlsbad, CA) and chloroform.

Total RNA (1 μg) served as template for cDNA synthesis in a reaction using 2 μl of random hexomers (0.5 μg/μl) with 200 U of Moloney murine leukemia virus-reverse transcriptase in a buffer containing 500 μM deoxynucleoside-5’-triphosphate (dNTP) and 10 mM dithiothreitol. cDNA samples were subjected to PCR in the presence of primers based on cloned rat COX isoforms (Tanaka et al., 2002). PCR, consisting of 5 μl of RT products, 2.5 U of Taq polymerase, 100 μM dNTP, and 0.5 μM primers, was carried out by a PCR-Express thermocycler (Hybaid, Ashford, Middlesex, UK). After 3 min at 94°C, the cycle conditions were 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for 30 cycles, followed by 7 min at 72°C. Aliquots of RNA not subjected to RT were included in PCR reactions to verify the absence of genomic DNA. The efficiency of RNA extraction, RT, and PCR was evaluated by primers for rat COX-2. PCR products were separated by 1.8% agarose gel electrophoresis in a Tris buffer (40 mM) containing 0.5 mM ethidium bromide. PCR products were then visualized by UV light and subjected to densitometric analysis by Kodak Image Station program (Eastman Kodak Co., Rochester, NY). The relative expression of target mRNA was normalized to that of β-actin.

Immunohistochemical Analysis. Specimens of colonic tissue, excised and fixed as reported above, were dehydrated with ethanol, treated with xylene, and embedded in paraffin at 56°C. Serial sections (5 μm thick) were processed for immunostaining. Slides were treated with 1% hydrogen peroxide in methanol, microwaved in citrate buffer, and blocked with normal swine serum (1:20; Dako-patts, Glosrup, Denmark). Sections were then incubated overnight at 4°C with the following polyclonal primary antibodies: rabbit anti-COX-1 (1:200; code 160109; Cayman Chemical Company, Ann Arbor, MI), rabbit anti-COX-2 (1:3000; code no. ALX 210711; Alexia Biochemicals, Lausen, Switzerland), and rabbit antineurofilament (1:400; Chemicon, code no. AB 1987, Temecula, CA). Immunoglobulins were diluted in PBS with 0.1% bovine serum albumin and 0.1% sodium azide. Sections were washed with PBS and incubated with biotinylated immunoglobulins followed by peroxidase-labeled streptavidin complex and 3,3’-diaminobenzidine tetrahydrochloride (DAB; Dakopatts) (Bernardini et al., 1999). Sections were counterstained with hematoxylin. All reactions were carried out at room temperature in a humidified chamber, and PBS was used for washes, unless otherwise specified. Negative controls were obtained by omitting primary antibodies or substituting the primary antibody with rabbit preimmune serum. Specificity of COX immunoreacting staining was assessed by preadsorbing anti-COX-1 and -COX-2 antibodies with COX-1 (code no. CAY 360109; Cayman Chemical Company) and COX-2 (code 160109; Cayman Chemical Company) blocking peptides, respectively, at 10 times the antibody concentrations for 24 h at 4°C. To test endogenous peroxidases and avidin-binding activity,
slides were incubated only with DAB or streptavidin-peroxidase complex plus DAB, respectively.

**Recording of Longitudinal Muscle Contractile Activity.** The contractile activity of colonic longitudinal smooth muscle was recorded as described previously by Blandizzi et al. (2003). Specimens of colon, excised as reported above, were placed into ice-cold preoxygenated Krebs’ solution, opened along the mesenteric insertion, and subjected to removal of mucosal/submucosal layer. The specimens were then cut along the longitudinal axis into strips of approximately 3-mm width and 20-mm length. The preparations were set up in 10-ml organ baths containing Krebs’ solution at 37°C, bubbled with 95% O₂ + 5% CO₂, connected to isotonic transducers (Basilie, Comerio, Italy) under a constant load of 1 g, and allowed to equilibrate for 30 min. Since there was no need to discriminate between active and passive tension developed by intestinal muscle, isotonic transducers were used to estimate changes in smooth muscle elongation under constant tension in response to stimulation. Krebs’ solution was changed at the following composition: 113 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, and 11.5 mM glucose, pH 7.4 ± 0.1. The contractile activity was recorded by a polygraph (Basilie). A pair of coaxial platinum electrodes was positioned at distance of 10 mm from longitudinal axis of each preparation to deliver TES by a BM-ST6 stimulator (Biomedica Mangoni, Pisa, Italy). Stimuli were applied as 10-s single trains of square wave pulses (0.5 ms, 30 mA, 10 Hz). Each preparation was repeatedly challenged with electrical stimulations, and experiments started when reproducible responses were obtained (usually after two to three stimulations).

In the first set of experiments, colonic preparations were exposed to indomethacin (COX-1/COX-2 inhibitor, 1 μM), 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole (SC-560; COX-1 inhibitor, 0.1 μM), or 5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfo- nyl)phenyl-2(5H)phenyl-2H)-furane (DFU; COX-2 inhibitor, 1 μM) for 30 min before transmural electrical stimulation (TES). Preparations were incubated with test drugs along two 15-min consecutive periods with an intervening washing. Drug concentrations were selected on the basis of previous studies (Riendeau et al., 1997; Kato et al., 2001).

The second set of experiments was designed to assay COX inhibitors on contractile responses elicited by TES directed to cholinergic innervation. For this purpose, colonic preparations were maintained in Krebs’ solution containing guanethidine (adrenergic blocker, 10 μM), N⁶-nitro-L-arginine methyl ester (L-NAME, inhibitor of nitric-oxide synthase, 100 μM), L-732,138 (NK₁ receptor antagonist, 10 μM), GR-159897 (NK₂ receptor antagonist, 1 μM), and SB-215795 (NK₃ receptor antagonist, 1 μM) to prevent noncholinergic motor responses (Fornai et al., 2005a). Incubation of colonic strips with COX inhibitors before challenge with TES was performed as reported above.

In the third series, COX inhibitors were assayed on cholinergic contractions elicited by direct pharmacological activation of muscarinic receptors on smooth muscle cells. Preparations were maintained in Krebs’ solution containing tetrodotoxin (1 μM) and stimulated twice with carbachol (muscarinic receptor agonist, 1 μM). The first stimulation was applied in the absence of other drugs, whereas the second one was applied after 30-min incubation with COX inhibitors, as reported above.

In a fourth set of experiments, the effects of COX inhibitors on TES-evoked motor responses were assessed on colonic preparations obtained from control or DNBS-treated rats treated daily with superoxide dismutase (SOD; 7 mg/kg/day s.c.) (Segui et al., 2004) or S-methylisothioura (SMT; 14 mg/kg/day s.c.), a selective inhibitor of inducible nitric-oxide synthase (iNOS) (Afulukwe et al., 2000), for 6 consecutive days starting 1 h before the induction of colitis.

**Drugs and Reagents.** Indomethacin, SC-560, atropine sulfate, hexamethonium bromide, N⁶-nitro-L-arginine methyl ester, carbachol hydrochloride, guanethidine, superoxide dismutase, and S-methylisothioura were from Sigma Chemical (St. Louis, MO); DFU was kindly provided by Merck Research Laboratories (Rahway, NJ); L-732,138, GR-159897, SB-215795, and tetrodotoxin were from Tocris Cookson (Bristol, UK); and random hexamers, Moloney murine leukemia virus-reverse transcriptase, Taq polymerase, and dNTP mixture were from Promega (Madison, WI). COX inhibitors were dissolved in dimethyl sulfoxide, and further dilutions were made with saline solution. Dimethyl sulfoxide concentration in organ bath never exceeded 0.5%.

**Statistical Analysis.** Results are given as mean ± S.E.M. The significance of differences was evaluated on raw data, prior to percentage normalization, by Student’s t test for unpaired data or one-way analysis of variance followed by post hoc analysis with Student-Newman-Keuls test, and P < 0.05 was considered significant. Colonic preparations included in each test group were obtained from distinct animals; therefore, the number of experiments refers also to the number of animals assigned to each group. Calculations were performed by commercial software (Prism, version 3.0; GraphPad Software Inc., San Diego, CA).

**Results**

**Assessment of Colitis.** Six days after DNBS treatment, the distal colon appeared thickened and ulcerated with evident areas of transmural inflammation. Adhesions were often present, and the bowel was occasionally dilated. Histologically, colitis was evident as granulocyte infiltration extending throughout the mucosa and submucosa, sometimes involving the muscular layer. More than 5-fold increase in both macroscopic and microscopic damage score was estimated in DNBS-treated animals in comparison with normal rats. In rats with colitis, treated with SOD or SMT, both macroscopic and microscopic damage scores were significantly decreased, although being still significantly greater than normal values (Table 1).

**RT-PCR Analysis.** RT-PCR analysis revealed the expression of COX-1 and COX-2 in colonic neuromuscular layers of both normal and DNBS-treated animals (Fig. 1A). The denitrosometric analysis, performed on amplified cDNA bands, indicated a significant increase in the expression of COX-2 mRNA in the presence of colitis (Fig. 1C), whereas no appreciable changes in the expression of COX-1 were detected (Fig. 1B).

**Immunohistochemical Analysis.** Both COX-1 and COX-2 were constitutively expressed in the tunica muscularis of normal rat colon (Fig. 2, A and C). In particular, COX-1 was found in neurons and glial cells of myenteric ganglia as

**TABLE 1**

Macroscopic and microscopic damage scores estimated for colon in rats under normal conditions or after treatment with DNBS, DNBS plus SOD, or DNBS plus SMT

<table>
<thead>
<tr>
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<th>Normal</th>
<th>DNBS</th>
<th>DNBS + SOD</th>
<th>DNBS + SMT</th>
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<tr>
<td>Macroscopic damage</td>
<td>1.56 ± 0.27</td>
<td>10.32 ± 1.44*</td>
<td>6.47 ± 1.60**</td>
<td>5.98 ± 1.15**</td>
</tr>
<tr>
<td>Microscopic damage</td>
<td>1.19 ± 0.31</td>
<td>7.86 ± 1.22*</td>
<td>4.11 ± 1.03**</td>
<td>4.89 ± 1.17**</td>
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Each value represents the mean of eight to 10 experiments ± S.E.M. Significant difference from the respective values obtained in normal rats: *, P < 0.05. Significant difference from the respective values obtained in animals treated with DNBS alone: †, P < 0.05.
well as in few cells of muscular layer (Figs. 2A and 3A), whereas little COX-2 immunoreactivity was expressed only in myenteric neurons (Figs. 2C and 3C). Colonic neuromuscular tissues from animals with colitis showed different patterns of COX isoform expression (Figs. 2, B and D, and 3, B and D) compared with normal rats. Myenteric ganglionic cells and few smooth muscle cells expressed detectable amount of COX-1 (Figs. 2B and 3B), which was similar to normal colonic tissues. In contrast, high COX-2 expression was found in the neuromuscular compartment of colonic specimens excised from animals with colitis; intense COX-2 immunoreactivity was detected in myenteric ganglionic cells and muscular layers (Figs. 2D and 3D). In the latter case, COX-2 was mainly localized at level of longitudinal muscle cell nuclei (Fig. 2D), in accordance with previously reported COX-2 nuclear labeling (Maihofner et al., 2000). Neurons of myenteric ganglia were localized by means of immunoreactivity to neurofilament both in normal (Fig. 2E) and inflamed (Fig. 2F) colon. Preadsorption of primary antibodies with the respective blocking peptides completely abolished any specific immunoreactivity (data not shown).

**Effects of COX Inhibitors on Longitudinal Smooth Muscle Activity.** During the equilibration period, most colonic preparations, obtained from normal or DNBS-treated rats, showed rapid and low in amplitude spontaneous activity, which remained stable throughout the experiment. TES-induced responses consisted of fast phasic contractions often followed by aftercontractions of variable amplitude (Fig. 4). The use of isotonic transducers allowed to record electrically evoked contractions as changes in smooth muscle elongation under constant tension, thus minimizing possible inflammation-induced variations of intrinsic contractile activity. Accordingly, control responses of normal tissues did not differ significantly from those observed in colonic preparations from DNBS-treated rats. Preincubation with atropine (1 μM) inhibited phasic contractions or converted them into relaxations, and only aftercontractions were evident. Tetrodotoxin (1 μM) abolished the contractile responses evoked by TES (not shown).

Incubation of colonic preparations from normal or DNBS-treated animals with indomethacin, SC-560 or DFU was not associated with significant changes in spontaneous motor activity. In normal colonic tissues, indomethacin significantly enhanced contractions evoked by TES (+64%, 1 μM) (Figs. 4 and 5A). SC-560 (0.1 μM) or DFU (1 μM) mimicked this enhancing action, but they were less effective than indomethacin (+41 and +36%) (Figs. 4 and 5A). After combined incubation with SC-560 plus DFU, TES elicited contractile responses with amplitude (+61%) comparable with that observed with indomethacin (Fig. 5A). In colonic preparations from rats with colitis, indomethacin (1 μM) or DFU (1 μM) evoked significant and similar increments of TES-induced contractions (+32 and +34%, respectively) (Figs. 4 and 5B), whereas SC-560 (0.1 μM) was without effect (Figs. 4 and 5B). After coincubation of colonic tissues with SC-560 plus DFU, the enhancement of TES-induced contractions was similar to that observed in the presence of indomethacin or DFU alone (+39%) (Fig. 5B).

In the presence of guanethidine, L-NAME, and NK receptor antagonists, TES evoked contractions of colonic preparations, obtained from normal or DNBS-treated rats, which were completely prevented, or markedly reduced, by atropine (1 μM, not shown). Under these conditions, TES-induced motor responses were suppressed by tetrodotoxin (1 μM) and unaffected by hexamethonium (10 μM). In normal preparations, indomethacin (1 μM) significantly enhanced cholinergic responses elicited by TES (+94%) (Fig. 5C). SC-560 (0.1 μM) or DFU (1 μM) mimicked this excitatory effect, although they were less effective (+58 and +61%) (Fig. 5C). Incubation with SC-560 plus DFU was followed by a potentiation of TES-induced contractions (+97%) similar to that achieved with indomethacin alone (Fig. 5C). In colonic tissues isolated from rats with colitis, indomethacin (1 μM) or DFU (1 μM) significantly increased TES-induced contractions and acted with similar efficacy (+55 and +49%, respectively), whereas SC-560 (0.1 μM) did not exert any significant effect (Fig. 5D). Following coincubation of colonic tissues with SC-560 plus DFU, TES-evoked contractions were enhanced to a similar extent to that observed with indomethacin or DFU alone (+64%) (Fig. 5D).

Exposure of colonic preparations, from normal or DNBS-treated animals, to carbachol (1 μM) resulted in phasic contractions (Fig. 6) sensitive to atropine. Under these conditions, carbachol-induced responses were not affected by indomethacin (1 μM) either in the absence or in the presence of colitis (Fig. 6). Similarly, SC-560 (0.1 μM), DFU (1 μM), or...
SC-560 plus DFU did not modify the motor responses elicited by carbachol (Fig. 6).

When colonic preparations, isolated from animals treated with DNBS plus SOD, were exposed to indomethacin (1 μM), the contractile responses evoked by TES were significantly increased (+66%) (Fig. 7A). Under these conditions, SC-560...
(0.1 μM) or DFU (1 μM) significantly enhanced TES-evoked motor activity, although they were less effective than indomethacin (28 and +46%, respectively) (Fig. 7A). Coincubation of colonic tissues with SC-560 plus DFU resulted in increments of TES-induced contractions similar to those observed in the presence of indomethacin alone (+69%) (Fig. 7A). In colonic tissues from rats with colitis subjected to in vivo iNOS blockade by SMT, COX inhibitors enhanced TES-induced contractions, with response patterns similar to those recorded after treatment of animals with DNBs plus SOD (Fig. 7B). Incubation of colonic tissues, obtained from normal rats treated with SOD or SMT, with COX inhibitors, resulted in enhancements of TES-induced contractions that did not differ significantly from those observed in the absence of SOD or SMT.

**Discussion**

This study provides evidence that both COX isoforms are constitutively expressed in colonic neuromuscular layers in the rat and contribute to the modulation of cholinergic motor activity acting at neuronal level and that in the presence of colitis, the COX-2 isoform plays a predominant role in this regulatory function. Our molecular and morphological analysis clearly demonstrated expression of both COX-1 and COX-2 in the neuromuscular layer of normal rat colon and up-regulation of COX-2 expression in the inflamed colon. In particular, immunohistochemistry indicated a predominant localization of both COX isoforms in neurons of myenteric ganglia and an increased immunostaining of COX-2 at the same sites in the presence of colitis. These findings add evidence to the concept that COX-2 can be expressed constitutively in the normal gut (as we and others have previously reported in human and mouse gut; Porcher et al., 2002, 2004; Fornai et al., 2005a) and could play a role in the control of myenteric nerve function during intestinal inflammation. In line with this view, Roberts et al. (2001) demonstrated increased COX-2 immunoreactivity in colonic myenteric neurons of patients with ulcerative colitis or Crohn’s disease, and...
Schwarz et al. (2001) found enhancement of COX-2 immunostaining in neurons and smooth muscle cells from jejunal tissues of rats undergoing intestinal manipulation (an experimental model of postoperative ileus).

The pharmacological blockade of COX isoforms in normal colonic preparations significantly increased electrically induced contractions, especially after pharmacological ablation of noncholinergic nerve pathways. Electrically evoked cholinergic contractions were enhanced after exposure to either SC-560 (COX-1 inhibitor) or DFU (COX-2 inhibitor). Even more pronounced effects (of similar magnitude) were recorded with the nonselective inhibitor indomethacin and with combined blockade of both COX isoforms by SC-560 plus DFU.

The present findings strengthen the notion that COX-derived products can modulate enteric cholinergic neurotransmission in the normal gut. De Backer et al. (2003) reported that indomethacin enhances the electrically induced acetylcholine release in muscle strips of pig stomach, suggesting inhibition by prostanoids of intramural cholinergic neurons. Furthermore, we previously observed that selective and nonselective COX inhibitors potentiate electrically induced cholinergic contractions of smooth muscle strips prepared from human distal colon (Fornai et al., 2005a). In contrast with findings in humans, pigs, and rats, experiments performed on guinea pig intestine indicated that the electrically or nicotine-induced acetylcholine release and related cholinergic contractions are enhanced by application of exogenous prostaglandins and inhibited by indomethacin, suggesting that in this species, endogenous prostanoids mediate excitation of cholinergic enteric pathways (Takeuchi et al., 1991). Therefore, the effects of prostanoids and COX inhibitors on gut motility depend on species, gut region, and muscular layer.

Our observation that TES- but not carbachol-induced contractions were affected by COX blockade indicates that the modulating control of COX pathways on cholinergic motor activity occurs at neuronal rather than at muscular sites. These functional data are consistent with our molecular analysis showing that both COX-1 and COX-2 were detected predominantly in neurons of myenteric ganglia, with no appreciable immunostaining in the muscular layers. Thus, it is conceivable that prostanoids produced by COX-1 and COX-2, both located in myenteric neurons, inhibit colonic cholinergic neurotransmission. In line with this proposal, preliminary in

**Fig. 6.** Top, representative tracings showing the contractile responses of longitudinal muscle preparations of colon obtained from normal rats or animals with DNBS-induced colitis. The colonic preparations were incubated in medium containing 1 μM tetrodotoxin and subjected to stimulation with 1 μM carbachol (CARB), either alone (CON) or in the presence of 1 μM indomethacin (IND). W, washing; CON, control contractions. Bottom, column graph showing the effects of 1 μM indomethacin (IND), 0.1 μM SC-560, 1 μM DFU, and SC-560 plus DFU on the contractile responses of longitudinal muscle preparations isolated from normal rats and exposed to 1 μM carbachol in the presence of 1 μM tetrodotoxin. Each column represents the mean ± S.E.M. value obtained from six experiments.

**Fig. 7.** Effects of 1 μM indomethacin (IND), 0.1 μM SC-560, 1 μM DFU, and SC-560 plus DFU on the contractile responses of longitudinal muscle preparations of colon obtained from rats with DNBS-induced colitis subjected to s.c. injection of 7 mg/kg/day superoxide dismutase (A) or 14 mg/kg/day S-methylisothiourea (B) for 6 days. The colonic preparations were incubated in standard medium and subjected to TES (0.5 ms, 10 Hz, 30 mA, 10 s). Each column represents the mean ± S.E.M. value obtained from 10 experiments. *P < 0.05; significant difference versus control value.
vivo experiments performed in our laboratory indicated that colonic transit in normal animals was enhanced by either COX-1 or COX-2 blockade, whereas COX-1 inhibition was without effect in rats with colitis (M. Forlani, L. Antonioli, R. Coluzzi, C. Blandizzi, and M. Del Tacca, unpublished data).

In colonic preparations obtained from rats with colitis, SC-560 lost its enhancing effect on electrically induced cholinergic contractions, whereas increments of similar degree were observed upon application of indomethacin, DFU, or indometacin plus DFU. The modulating function of COX-2 was likely to occur still at neuronal level because neither indomethacin nor DFU affected carbachable-induced contractions.

An intriguing finding in rats with colitis is that the functional data are not fully consistent with the patterns of COX isofrom localization; although expression of COX-2 increased, the motor enhancing effects resulting from its pharmacological inhibition remained nearly unchanged. On the other hand, COX-1 expression did not vary, but its blockade was without effects on the evoked cholinergic contractions. To get further insight into this aspect, it is noteworthy that gut inflammation is associated with increased production of reactive oxygen species, such as peroxide anion radicals (O₂⁻) and peroxynitrite anions (ONOO⁻), known to induce oxidative tissue injury (Dijkstra et al., 1998), and that peroxynitrite anions, generated from the reaction between nitric oxide (NO) and superoxide anions, can down-regulate the catalytic activities of COX enzymes, especially COX-1 (Fujimoto et al., 2004). Accordingly, antioxidants were reported to ameliorate experimental colitis (Segui et al., 2004; Oz et al., 2005). Thus, it is conceivable that, in the presence of colitis, increased production of peroxynitrite anions could functionally inactivate COX-1 activity, whereas COX-2 overexpression could remain functionally silent because of the inhibition exerted by reactive oxygen species. To test this hypothesis, we performed functional experiments on colonic preparations obtained from rats with colitis treated with SOD (the enzyme responsible for O₂⁻ inactivation) or SMT (a selective inhibitor of iNOS, the main source of NO in inflamed tissues). Thus, evidence was obtained that the in vivo antioxidant treatment with SOD or SMT could prevent COX-1 inactivation. Indeed, under these conditions, SC-560 almost completely recovered its ability to enhance the electrically evoked contractions of colonic longitudinal muscle strips.

Taking together our molecular and pharmacological findings, it appears that, in the presence of inflammation, changes in the control of colonic motility by COX pathways reflect mainly impaired function of these enzymes because of increased oxidative stress. Thus, as postulated by Costa (2004), COX-derived mediators do not seem to play distinct physiological and pathological roles, at least in the case of gut motility, and the induction of COX-2 expression by intestinal inflammation might be viewed as an attempt of endogenous homeostatic mechanisms to preserve or restore the modulating actions of COX products.

In conclusion, this study indicates that both COX-1 and COX-2 are constitutively expressed in the neuromuscular layer of normal rat colon, where they are mainly localized in neurons of myenteric ganglia and contribute to inhibition of excitatory cholinergic pathways. In the presence of colitis, the COX-2 isoform seems to play a predominant role in driving this modulating function.

References

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