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Differential role of cyclooxygenase 1 and 2 isoforms in the modulation of colonic neuromuscular function in experimental inflammation

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Abbreviations: COX, cyclooxygenase; DAB, 3,3'-diaminobenzidine tetrahydrochloride; DNBS, 2,4-dinitrobenzenesulfonic acid; dNTP, deoxynucleotide triphosphate; L-NAME, N^{ω}-nitro-L-arginine methylester; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; PBS, phosphate buffered saline; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SEM, standard error of mean; SMT, S-methylisothiourea; SOD, superoxide dismutase; TES, transmural electrical stimulation.

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ABSTRACT

This study examines the role played by cyclooxygenase isoforms (COX-1 and COX-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 RT-PCR 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 upregulated in the presence of colitis. In normal and inflamed colon, both COX isoforms were mainly localized in neurones of myenteric ganglia. In the normal colon, indomethacin (COX-1/COX-2 inhibitor), SC-560 (COX-1 inhibitor) or DFU (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 non-cholinergic 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 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.

Introduction

Since the discovery of two cyclooxygenase (COX) isoforms (COX-1, 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 (Fornai et al., 2005a; Wallace and Devchand, 2005), whereas in the presence of ulcer, inflammatory or neoplastic diseases, COX-2 expression can be upregulated. 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 non-steroidal anti-inflammatory drugs (NSAIDs) (Tanaka et al., 2002), and the carcinogenesis and tumour cell proliferation promoted by COX-2-derived prostanoids (Subbaramaiah and Dannenberg, 2003). By 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; Fornai et al., 2005b), 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; Porcher et al., 2004; Fornai et al., 2005b), 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 neurones. In this model, the inhibition of COX-2, but not COX-1, restored the normal electrical properties of AH neurones, whereas the application of prostaglandin E_2 to inflamed colonic preparations decreased the afterhyperpolarization of AH neurones 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 non-selective COX-inhibitors.

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 50-60% 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 previously described by Barbara et al. (2000). Briefly, during anaesthesia with diethyl ether, 30 mg of 2,4-dinitrobenzenesulphonic acid (DNBS) in 0.25 ml of 50% ethanol were administered intrarectally via a polyethylene PE-60 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 & 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 faecal material (indirect marker of diarrhoea); thickening of colonic wall; presence and extension of hyperaemia and macroscopic mucosal damage (assessed with the aid of a ruler). Microscopic criteria were assessed by light microscopy on haematoxylin- 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-polymerase chain reaction (RT-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, U.S.A.) and chloroform. Total RNA (1 µg) served as template for cDNA synthesis in a reaction using 2 µl random hexamers (0.5 µg/µl) with 200 U of MMLV-reverse transcriptase in a buffer containing 500 µM deoxynucleotide triphosphate mixture (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, Taq polymerase 2.5 U, dNTP 100 µM and primers 0.5 µM, was carried out by a PCR-Express thermocycler (Hybaid, Ashford, Middlesex, U.K.). 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 β-actin. PCR products were separated by 1.8% agarose gel electrophoresis in a Tris buffer 40 mM containing 2 mM ethylenediamine tetraacetic acid, 20 mM acetic acid (pH 8), and stained with 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, U.S.A.). 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; Dakopatts, Glostrup, Denmark). Sections were then incubated overnight at 4°C with the following polyclonal primary antibodies: rabbit anti-COX-1 (1:200; Cayman Chemical Company, code no. 160109, Ann Arbor, Michigan, U.S.A.); rabbit anti-COX-2 (1:3000; Alexis Biochemicals, code no. ALX 210711, Lausen, Switzerland); rabbit anti-neurofilament (1:4000; Chemicon, code no. AB 1987, Temecula, California, U.S.A.). 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, Glostrup, Denmark) (Bernardini et al., 1999). Sections were counterstained with haematoxylin. 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 pre-adsorbing anti-COX-1 and -COX-2 antibodies with COX-1 (Cayman Chemical Company, cod. no. CAY 360109) and COX-2 (Alexis Biochemicals, cod. no. ALX 153-063) blocking peptides, respectively, at ten 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 previously described by Blandizzi et al. (2003). Specimens of colon, excised as reported above, were placed into cold pre-oxygenated 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% O_2+5% CO_2, connected to isotonic transducers (Basile, 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 had the following composition (mM): NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.5 (pH 7.4±0.1). The contractile activity was recorded by a polygraph (Basile, Comerio, Italy). A pair of coaxial platinum electrodes was positioned at distance of 10 mm from longitudinal axis of each preparation to deliver transmural electrical stimulation (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 2-3 stimulations).

In the first set of experiments, colonic preparations were exposed to indomethacin (COX-1/COX-2 inhibitor, 1 μM), SC-560 (COX-1 inhibitor, 0.1 μM) or DFU (COX-2 inhibitor, 1 μM), for 30 min before 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).

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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), N^{ω}-nitro-L-arginine methylester (L-NAME, inhibitor of nitric oxide synthase, NOS, 100 μM), L-732,138 (NK_1 receptor antagonist, 10 μM), GR-159897 (NK_2 receptor antagonist, 1 μM) and SB-218795 (NK_3 receptor antagonist, 1 μM), to prevent non-cholinergic motor responses (Fornai et al., 2005b). 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, while 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-methylisothiourea (SMT, 14 mg/kg/day s.c.), a selective inhibitor of inducible NOS (iNOS) (Afulukwe et al., 2000), for 6 consecutive days starting 1 h before the induction of colitis.

Drugs and reagents

Indomethacin, SC-560 ([5-(4-clorophenyl)-1-(4-metoxyphenyl)-3-trifluoromethyl-pirazole), atropine sulphate, hexamethonium bromide, N^{ω}-nitro-L-arginine methylester, carbachol hydrochloride, guanethidine, superoxide dismutase, S-methylisothiourea (Sigma Chemical, St. Louis, MO, U.S.A.); DFU [3-(3-fluorophenyl)-4-(4-methanesulfonyl)-5,5-dimethyl-5H-furan-2-one] (kindly provided by Merck Research Laboratories, Rahway, NJ,

U.S.A.); L-732,138 (N-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzyl ester), GR-159897 (5-fluoro-3-[2-[4-methoxy-4-[[(R)-phenylsulphinyl]methyl]-1-piperidinyl]ethyl]-1H-indole), SB-218795 ((R)-[[2-phenyl-4-quinolinyl)carbonyl]amino]-methyl ester benzeneacetic acid), tetrodotoxin (Tocris Cookson, Bristol, U.K.); random hexamers, MMLV-reverse transcriptase, *Taq* polymerase, dNTP mixture (Promega, Madison, WI, U.S.A.). COX inhibitors were dissolved in dimethylsulphoxide and further dilutions were made with saline solution. Dimethylsulphoxide concentration in organ bath never exceeded 0.5%.

Statistical analysis

Results are given as mean standard ± error of mean (SEM). The significance of differences was evaluated on raw data, prior to percentage normalization, by Student *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, and therefore the number of experiments refers also to the number of animals assigned to each group. Calculations were performed by commercial software (GraphPad Prism™, version 3.0 from GraphPad Software Inc., San Diego, CA, U.S.A.).

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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 five-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 (Figure 1A). The densitometric analysis, performed on amplified cDNA bands, indicated a significant increase in the expression of COX-2 mRNA in the presence of colitis (Figure 1C), whereas no appreciable changes in the expression of COX-1 were detected (Figure 1B).

Immunohistochemical analysis

Both COX-1 and COX-2 are constitutively expressed in the tunica muscularis of normal rat colon (Figure 2A and C). In particular, COX-1 was found in neurons and glial cells of myenteric ganglia as well as in few cells of muscular layer (Figure 2A and 3A), whereas little COX-2 immunoreactivity was expressed only in myenteric neurons (Figure 2C and 3C). Colonic neuromuscular tissues from animals with colitis showed different patterns of COX isoform expression (Figure 2B, D and 3B, D) compared to normal rats. Myenteric ganglionic cells and few smooth muscle cells expressed detectable amount of COX-1 (Figure 2B and 3B), which was similar to normal colonic tissues. By 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 (Figure 2D and 3D). In the latter case, COX-2 was mainly localized at level of longitudinal muscle cell nuclei (Figure 2D), in accordance with previously reported COX-2 nuclear labelling (Maihofner et al., 2000). Neurones of myenteric ganglia were localized by means of immunoreactivity to neurofilament both in normal (Figure 2E) and inflamed colon (Figure 2F). Pre-adsorption 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 (Figure 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. Pre-incubation 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) (Figure 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%)

(Figure 4 and 5A). After combined incubation with SC-560 plus DFU, TES elicited contractile responses with amplitude (+61%) comparable to that observed with indomethacin (Figure 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) (Figure 4 and 5B), whereas SC-560 (0.1 μM) was without effect (Figure 4 and 5B). After co-incubation 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%) (Figure 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%) (Figure 5C). SC-560 (0.1 μM) or DFU (1 μM) mimicked this excitatory effect, although they were less effective (+58% and +61%) (Figure 5C). Incubation with SC-560 plus DFU was followed by a potentiation of TES-induced contractions (+97%) similar to that achieved with indomethacin alone (Figure 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 (Figure 5D). Following co-incubation 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%) (Figure 5D).

Exposure of colonic preparations, from normal or DNBS-treated animals, to carbachol (1 μM) resulted in phasic contractions (Figure 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 (Figure 6). Analogously, SC-560 (0.1 μM), DFU (1 μM) or SC-560 plus DFU did not modify the motor responses elicited by carbachol (Figure 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%) (Figure 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) (Figure 7A). Co-incubation 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%) (Figure 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 (Figure 7B). Incubation of colonic tissues, obtained from normal rats treated with SOD or SMT, with COX inhibitors, resulted in enhancements of TES-induced contractions which did not differ significantly from those observed in the absence of SOD or SMT.

Discussion

This study provides evidence that: 1) 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; 2) 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; Fornai et al., 2005b; Porcher et al., 2002; Porcher et al., 2004) and could play a role in the control of myenteric nerve function during intestinal inflammation. In addition, 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 neurones 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 non-cholinergic 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 non selective 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 neurones. Furthermore, we previously observed that selective and non selective COX inhibitors potentiate electrically induced cholinergic contractions of smooth muscle strips prepared from human distal colon (Fornai et al., 2005b). 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 neurones 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 neurones, inhibit colonic cholinergic neurotransmission. In line with this proposal, preliminary *in 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 effects in rats with colitis (Del Tacca et al., 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 indomethacin plus DFU. The modulating function of COX-2 was likely to occur still at neuronal level, as neither indomethacin nor DFU affected carbachol-induced contractions.

An intriguing finding in rats with colitis is that the functional data are not fully consistent with the patterns of COX isoform localization: while 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 (ROS), such as superoxide anion radicals (O_2^-) 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 downregulate 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 O_2^- inactivation) or SMT (a selective inhibitor of iNOS, the main source of NO in inflamed tissues). Evidence was thus 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 neurones 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 action.

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Footnotes

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Figure legends

Figure 1. Reverse transcription-polymerase chain reaction analysis of COX-1, COX-2 and β-actin mRNA in the muscular layer of distal colon obtained from rats under normal conditions or with DNBS-induced colitis. (A) Three representative agarose gels, referring to the amplification of COX-1, COX-2 and β-actin cDNAs. (B, C) Column graphs referring to the densitometric analysis of COX-1 and COX-2 cDNAs bands normalised to the expression of β-actin. Each column represents the mean value ± SEM obtained from 6 experiments. **P*<0.05: significant difference *vs* normal values.

Figure 2. Immunohistochemical detection of COX-1 (A, B), COX-2 (C, D) and neurofilament (NFL; E, F) performed on adjacent sections of colon from rats under normal conditions (A, C, E, G) or with DNBS-induced colitis (B, D, F, H). Circular (cm) and longitudinal (lm) smooth muscle. Neurons of myenteric ganglia (arrowheads). (A) Normal colonic neuromuscular tissue shows detectable amount of COX-1 in neurons (arrowheads) and glial cells of myenteric ganglia as well as in few smooth muscle cells of circular and longitudinal muscular layers. (B) Rats with colitis show no appreciable variation of COX-1 expression in colonic neuromuscular structures compared to normal rats. (C) COX-2 is weakly expressed in myenteric neurons (arrowheads) of colon from normal animals. (D) COX-2 staining is enhanced in colonic neuromuscular structures of rats with colitis: COX-2 is strongly expressed both in myenteric ganglia (arrowheads) and tunica muscularis mainly at level of longitudinal layer. (E, F) Neurons (arrowheads) are identified within myenteric neurons of colon from normal (E) or DNBS-treated (F) rats by anti-neurofilament (NFL) immunoreaction. (G, H) No specific immunoreaction is observed in serial sections of specimens from normal (G) or DNBS-treated (H) animals, incubated with preimmune rabbit serum.

Figure 3. Higher magnification of myenteric ganglia represented in boxed areas of Figure 2: COX-1 (A, B) and COX-2 (C, D) were detected in colon from normal (A, C) and DNBS-treated (B, D) rats. Neurons of myenteric ganglia (arrows). Neurons were immunostained for COX-1 both in normal (A) and inflamed (B) rat colon without appreciable differences in cytoplasmatic immunostaining. The weak COX-2 signal observed in myenteric neurons from normal rats (C) was enhanced in neurons and glial cells of myenteric ganglia of DNBS-treated rats (D). In rats with colitis, COX-2 immunostaining was observed also in muscle cell nuclei of longitudinal layer (arrowheads).

Figure 4. Representative tracings showing the contractile responses of longitudinal muscle preparations of colon obtained from normal rats (upper panel) or animals with DNBS-induced colitis (lower panel). The colonic preparations were incubated in standard medium and subjected to transmural electrical stimulation (TES: 0.5 ms, 10 Hz, 30 mA, 10 s) either alone (CON) or in the presence of indomethacin 1 μM (IND), SC-560 0.1 μM or DFU 1 μM. W, washing; TES, transmural electrical stimulation; CON, control contractions.

Figure 5. Effects of indomethacin (IND, 1 μM), SC-560 0.1 μM, DFU 1 μM and SC-560 plus DFU on the contractile responses of longitudinal muscle preparations of colon, obtained from normal rats (A, C) or animals with DNBS-induced colitis (B, D), under the following conditions: (A, B) incubation in standard medium and application of transmural electrical stimulation (TES: 0.5 ms, 10 Hz, 30 mA, 10 s); (C, D) incubation in medium containing guanethidine 10 μM, L-NAME 100 μM, L-732,138 10 μM, GR-159897 1 μM, SB-218795 1 μM, and application of TES. Each column represents the mean ± SEM value obtained from 8-10 experiments. *P<0.05: significant difference *vs* control value.

Figure 6. Upper panel: 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 tetrodotoxin 1 μM and subjected to stimulation with carbachol 1 μM (CARB), either alone (CON) or in the presence of indomethacin 1 μM (IND). W, washing; CON, control contractions. Lower panel: column graph showing the effects of indomethacin (IND, 1 μM), SC-560 0.1 μM, DFU 1 μM and SC-560 plus DFU on the contractile responses of longitudinal muscle preparations isolated from normal rats and exposed to carbachol 1 μM in the presence of tetrodotoxin 1 μM. Each column represents the mean ± SEM value obtained from 6 experiments.

Figure 7. Effects of indomethacin (IND, 1 μM), SC-560 0.1 μM, DFU 1 μM and SC-560 plus DFU on the contractile responses of longitudinal muscle preparations of colon obtained from rats with DNBS-induced colitis subjected to subcutaneous injection of superoxide dismutase 7 mg/kg/day (A) or S-methylisothiourea 14 mg/kg/day (B) for six days. The colonic preparations were incubated in standard medium and subjected to transmural electrical stimulation (TES: 0.5 ms, 10 Hz, 30 mA, 10 s). Each column represents the mean ± SEM value obtained from 10 experiments. *P<0.05: significant difference *vs* control value. SOD, superoxide dismutase; SMT, S-methylisothiourea.

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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.

	Normal	**DNBS**	**DNBS+SOD**	**DNBS+SMT**
Macroscopic damage	1.56±0.27	10.32±1.44*	6.47±1.60 *,#	5.98±1.15 *,#
Microscopic damage	1.19±0.31	7.86±1.22*	4.11±1.03 *,#	4.89±1.17 *,#

Each value represents the mean of 8-10 experiments ± SEM. 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.

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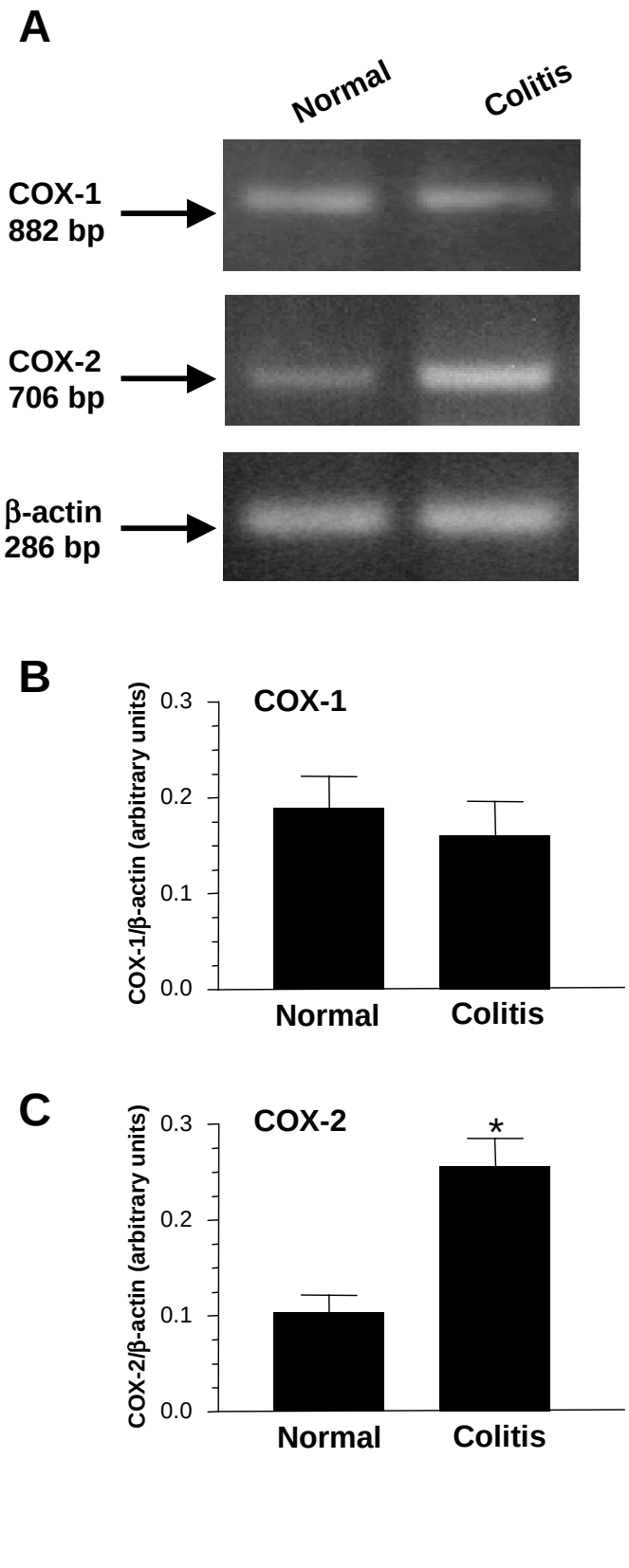

Figure 1

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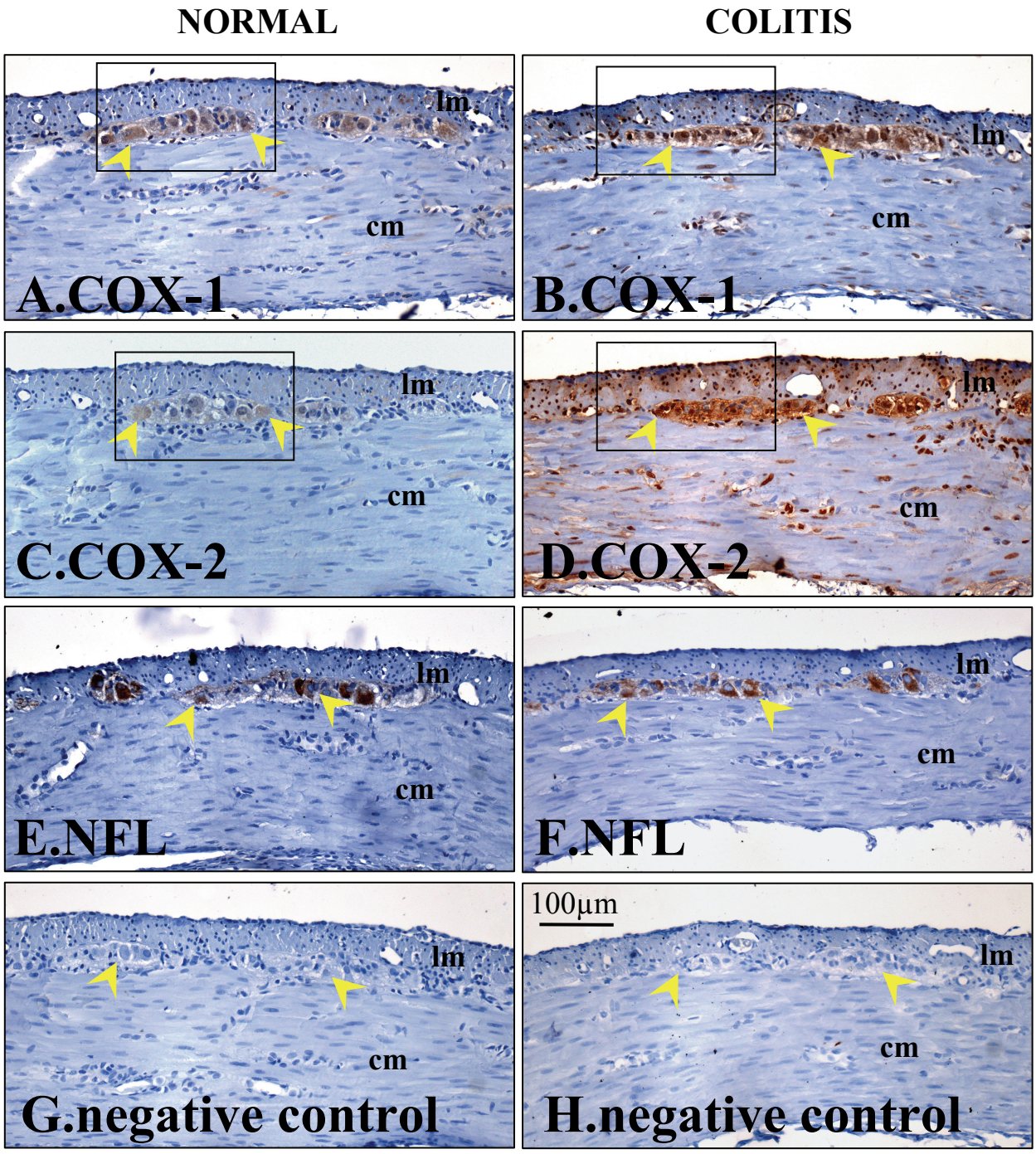

Figure 2

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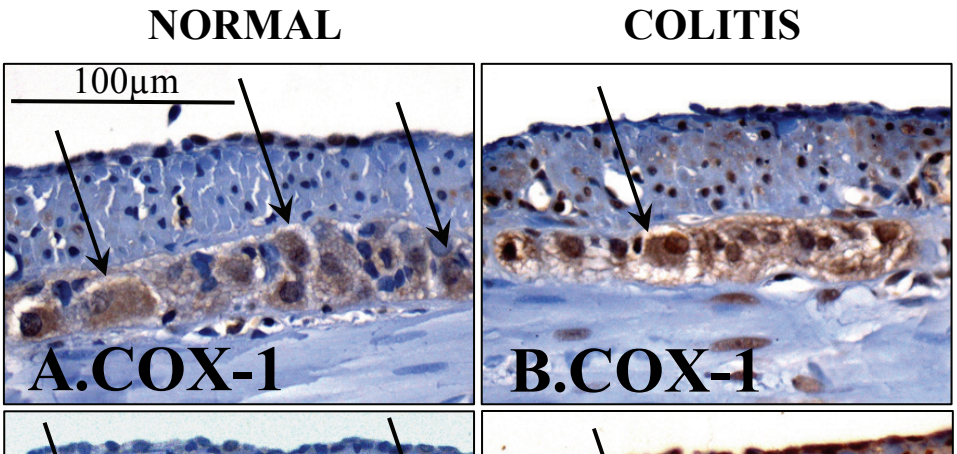

Figure 3

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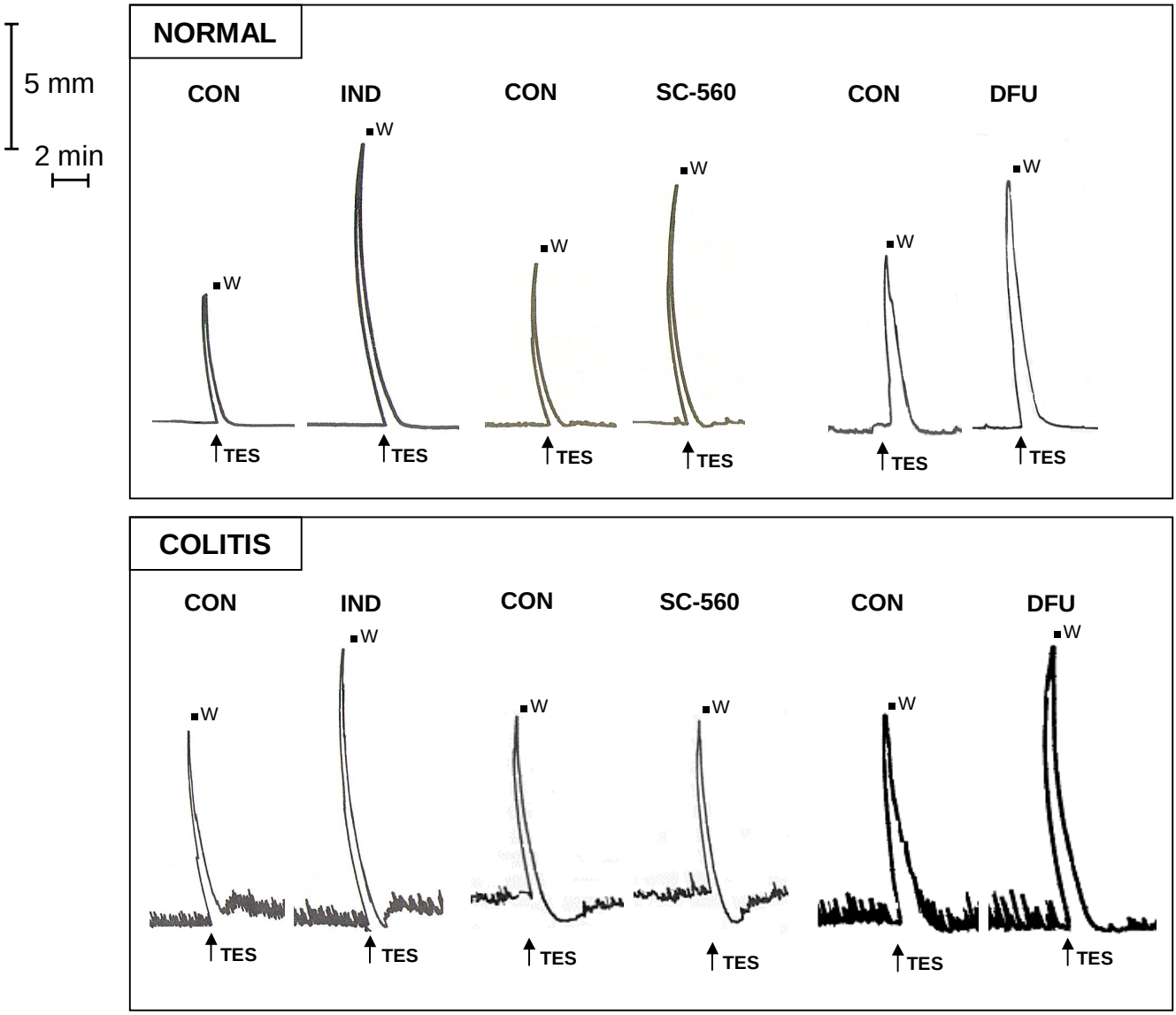

Figure 4

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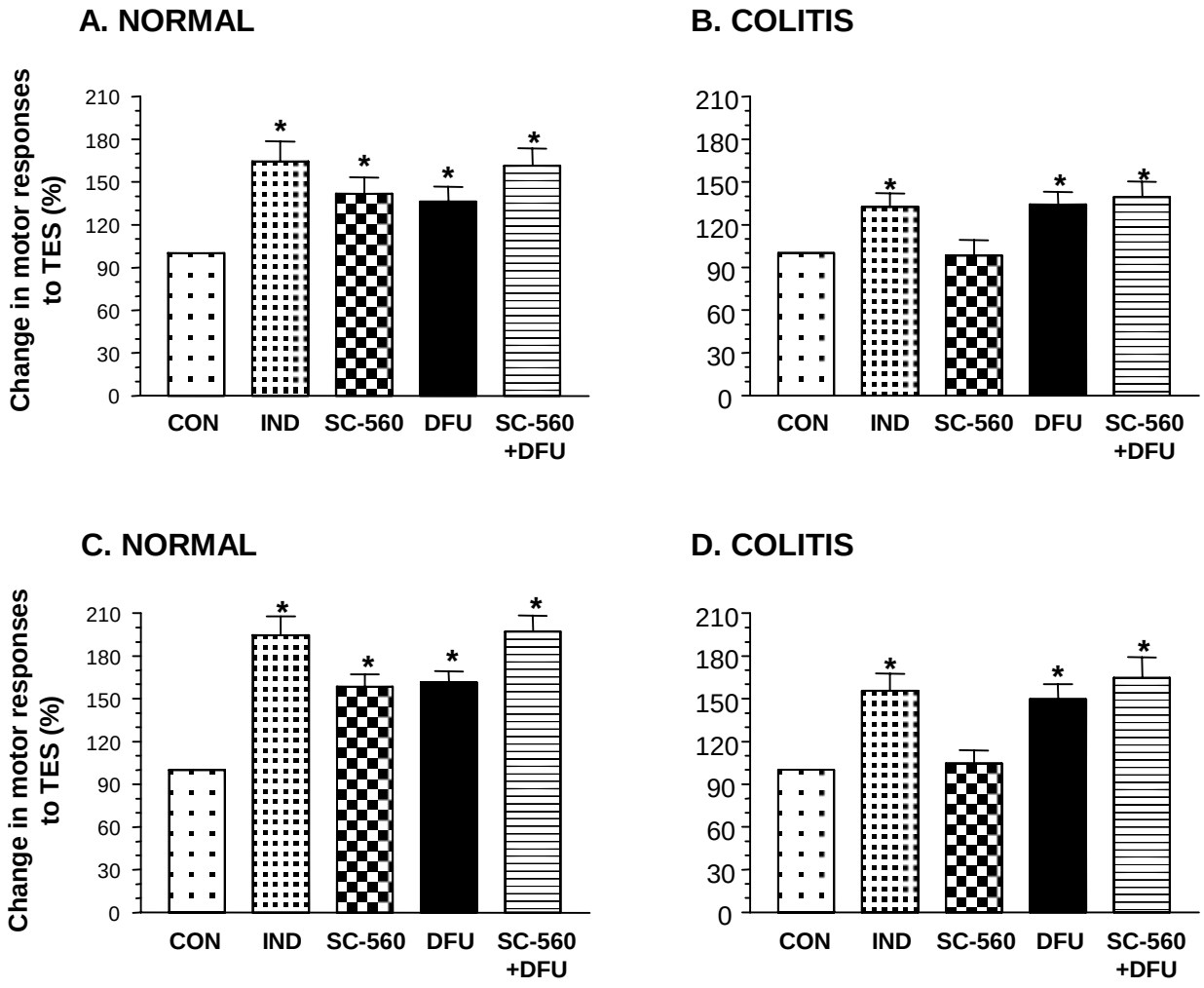

Figure 5

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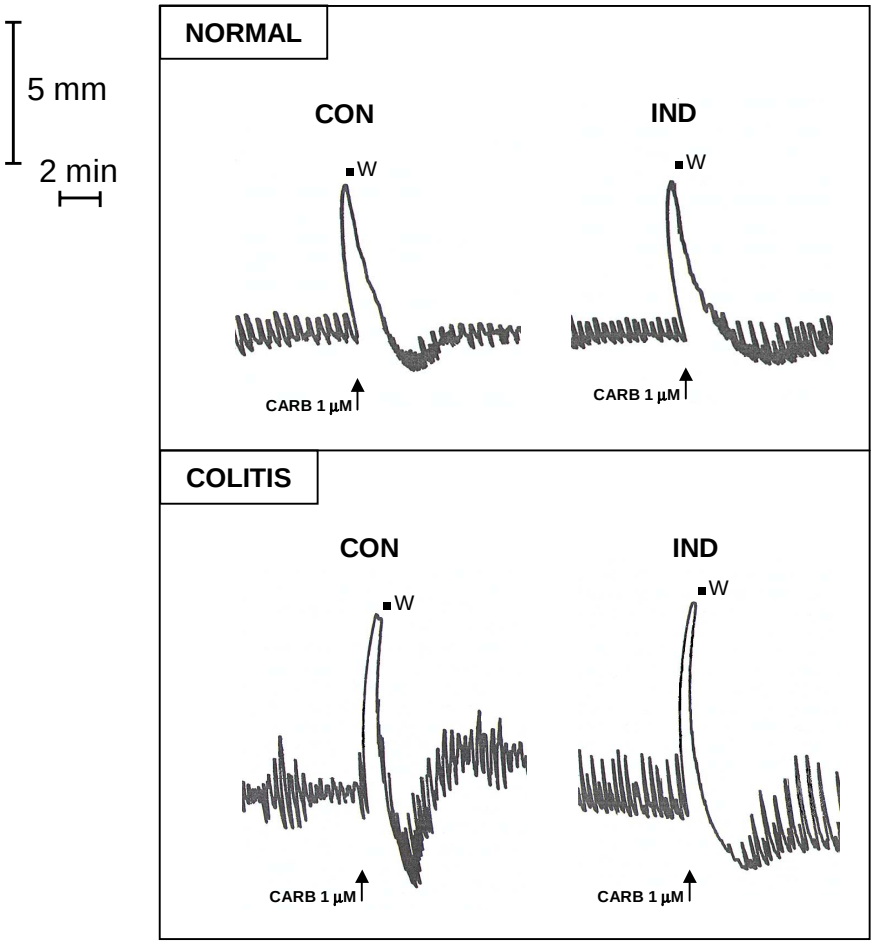

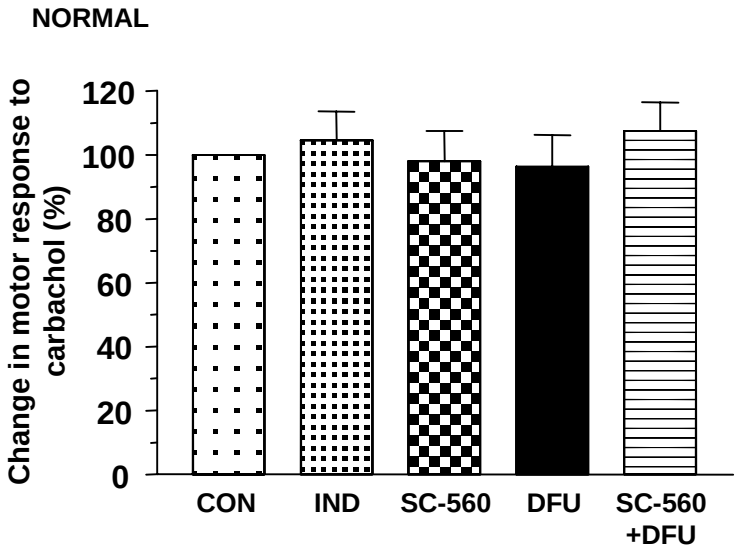

Figure 6

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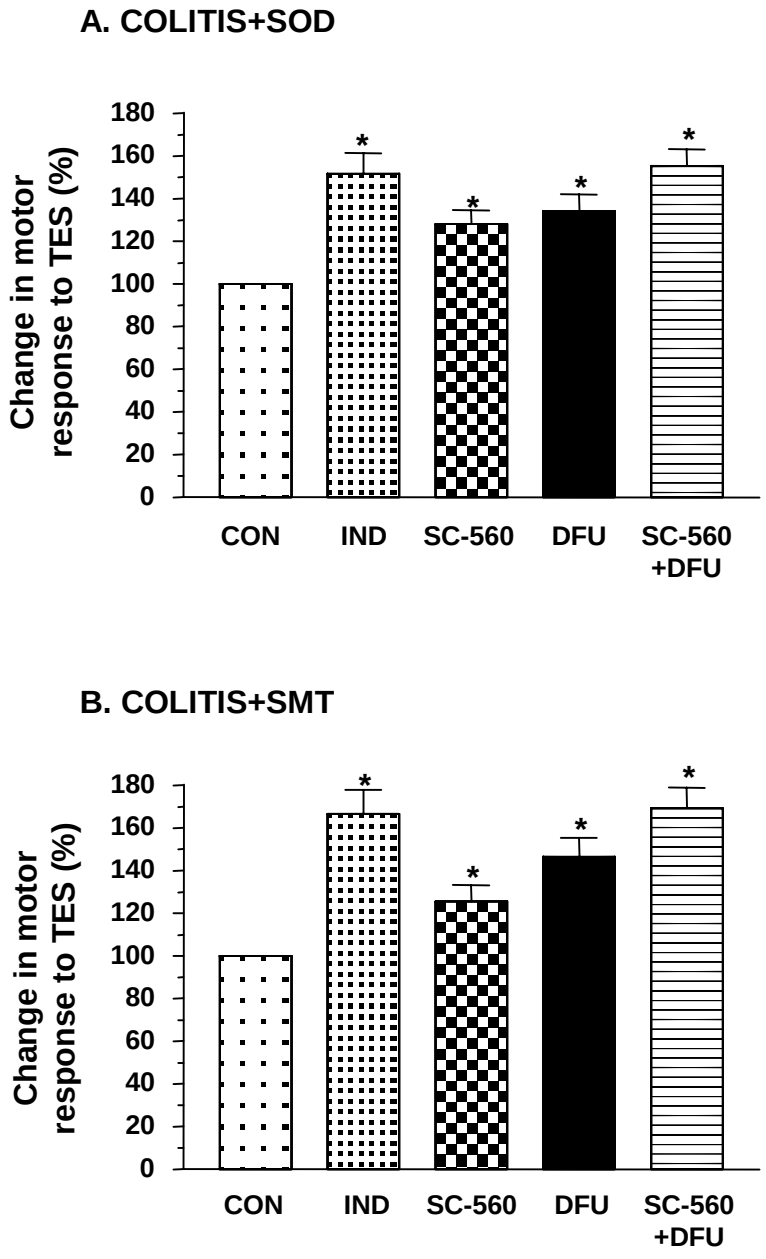

Figure 7