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Research Paper

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Role of cyclooxygenase pathways in bowel fibrotic remodelling in a murine model of experimental colitis

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Abstract

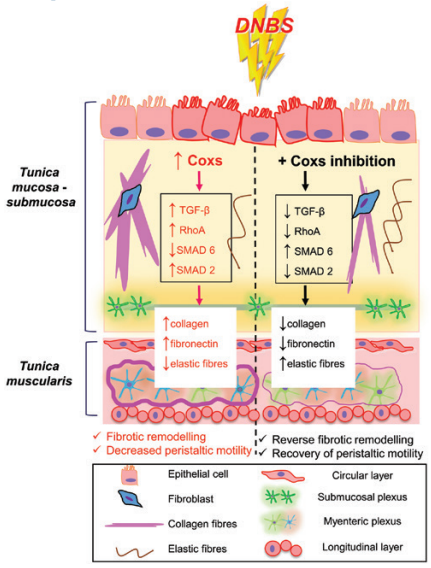
Objective Gut fibrosis occurs under chronic inflammation. This study examined the effects of different cyclooxygenase (COX) inhibitors on fibrosis in the inflamed colon.

Methods Colitis was induced by 2,4-dinitrobenzenesulfonic acid (DNBS) in albino male Sprague–Dawley rats. After 6, 12 and 18 days, macroscopic and microscopic damage, collagen and elastic fibre content were examined. At day 6, pro-fibrotic factors (collagen I and III, hydroxyproline, fibronectin, matrix metalloproteinase-2 and -9), transforming growth factor-beta (TGF-β) signalling [TGF-β, Ras homolog gene family member A (RhoA), phosphorylated small mother against decapentaplegic (pSMAD)-2 and -6] and peristalsis were assessed, and the effects of indomethacin, SC-560 or celecoxib were tested.

Key findings Six days after DNBS administration, significant histopathological signs of fibrotic remodelling were observed in rats. At day 6, pro-fibrotic factors were up-regulated and colonic peristalsis was altered. COX inhibitors reversed the histochemical, molecular and functional changes in the fibrotic colon. COX inhibition reduced TGF-β expression, SMAD2 phosphorylation and RhoA, and increased SMAD6 expression.

Conclusions Colonic fibrosis is associated with altered bowel motility and induction of profibrotic factors driven by TGF-β signalling. COX-1 and COX-2 inhibition counteracts this fibrotic remodelling by the modulation of TGF-β/SMAD signalling, mainly via SMAD6 induction and reduction in SMAD2 phosphorylation.

Graphical Abstract



Keywords: Cyclooxygenase; fibrosis; inflammatory bowel disease; SMAD; TGF-β

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Introduction

Inflammatory bowel diseases (IBDs) include chronic pathological conditions characterized by periods of relapsing disease activity and remission that, over the time, can lead to severe tissue damage up to abnormal repair and fibrosis of the intestinal wall. Both Crohn's disease and ulcerative colitis, although with different incidence and tissue patterns, can be exacerbated by intestinal fibrotic deposition. Intestinal fibrosis can contribute to gut motor dysfunctions and progress up to the formation of strictures, with consequent clinical symptoms of bowel obstruction,[1, 2] impaired quality of life and increased risk of surgical interventions.[1–3]

Among the pharmacological approaches able to counteract fibrosis in other tissues, non-steroidal anti-inflammatory drugs (NSAIDs), have demonstrated some efficacy in the control of hepatic[4, 5] and renal tubulointerstitial fibrosis.[6] NSAIDs prevent the formation of prostanoids, including prostaglandin (PG) E_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A2, from arachidonic acid through the inhibition of cyclooxygenase (COX) enzymes.[7]

Moreover, COX-2 inhibitors have been reported to be effective against intra-abdominal adhesions through a decrease in the activity of peritoneal fibroblasts.[8] Taken together, these observations sustain the hypothesis that COX pathways might play a relevant role in the pathophysiology of tissue fibrotic remodelling in different body districts, including the digestive tract. In this context, it is noteworthy that COX-derived PGE_2 plays a dual role since it is involved both in the maintenance of gut epithelium, protecting against acute damage and facilitating regeneration after injury,[9–12] and in shaping immune/inflammatory responses during inflammation through EP4 receptors.[13]

Based on the above background, this study was designed to evaluate the impact of pharmacological COX inhibition on the molecular events and morphological patterns associated with the intestinal fibrotic remodelling in the setting of experi-mental bowel inflammation. This knowledge provides new insights about the role of COXs in gut fibrotic processes and impaired bowel propulsive motility associated with bowel inflammation, thus allowing to identify novel pharmacological targets for potential therapeutic interventions.

Materials and Methods

Animals

Albino male Sprague–Dawley rats (200–250 g body weight, 8 weeks of age, from ENVIGO, Udine, Italy) were housed in a 12 h light/dark cycle at 22–24°C and 50–60% humidity, as described previously.[14] Animal care and handling were in line with the European Community Council Directive 2010/63/UE, recognized and adopted by the Italian Government. Ethical Committee of the University of Pisa and the Italian Ministry of Health (authorization n° 674/2016-PR, 2016) approved all experiments.

Induction and assessment of colitis

2,4-Dinitrobenzenesulfonic acid (DNBS) 30 mg in 0.25 ml of 50% ethanol, was intrarectally administered in rats to induce colitis, as described previously.[14] Control rats (normal) received 0.25 ml of 50% ethanol. Animals were euthanized 6, 12 and 18 days after DNBS administration (Figure 1), to characterize the timing required to detect fibrotic changes.

Two operators blinded to treatments evaluated macroscopic and microscopic colonic damage, as previously reported.[14] The macroscopic damage was scored for each rat on a 0- to 6-point scale, as displayed in Table 1.

The microscopic damage was scored on haematoxylin/eosin-stained cross-sections of colonic samples and based on the following parameters: (1) mucosal architecture loss (0–3); (2) goblet cell depletion (0, absent; 1, present); (3) crypt abscess (0, absent; 1, present); (4) cellular infiltration (0–3); (5) *tunica muscularis* thickening (0–3).

Evaluation of colonic fibrosis

The presence of collagen and elastic fibres within the colonic wall was detected on full-thickness colonic cross-sections by Sirius red-fast green and orcein histochemical staining, respectively, as previously described.[15] Positive-stained areas were calculated by Image Analysis System of Leica Application Suite (L.A.S.) software v.4 and extrapolated histochemical data were expressed as ratio between percentage of positive pixels and total tissue area examined.

The amount of collagen deposition was evaluated further by assessment of tissue hydroxyproline levels, as previously described.[16]

Drug treatments

Since at day 6 the presence of fibrosis was well evident, animals (n = 6/group) were treated daily: indomethacin (non-selective COX inhibitor, 2 mg/kg/day), SC-560 (selective COX-1 inhibitor, 2.5 mg/kg/day), celecoxib (selective COX-2 inhibitor, 1 mg/kg/day) or carboxymethylcellulose (vehicle, 3%) orally for three days starting three days after DNBS administration (Figure 1). COX inhibitor doses were chosen on the basis of previous experiences.[17, 18]

Western blot analysis

Colonic tissue specimens were homogenized in radioimmunoprecipitation assay buffer and centrifuged at 20,000 rpm for 15 min at 4°C.[17] The supernatants were separated from pellets and stored at –20°C. Aliquots of 50 µg of protein were separated by electrophoresis on 8% sodium dodecylsulfate-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. Then, the experimental steps were: (1) blocking for 1 h with 5% non-fat dried milk in Tween-20; (2) incubation at room temperature (RT) with primary antibodies anti-collagen-I, -III (1:5000, Abcam, Cambridge, UK), transforming growth factor-beta (TGF-β) (1:500, Abcam, Cambridge, UK), anti-fibronectin (1:10 000, Epitomics, CA, USA); anti-metalloproteinase (MMP)-2 and -9 (1:1000, Calbiochem, UK); anti-Ras homolog gene family, member A (RhoA) (1:1000, Cytoskeleton, CO, USA); anti-phospho-small mother against decapentaplegic 2 (SMAD2) (1:1000, Cell Signalling Technology, USA); anti-SMAD6 (1:1000, Cayman Chemical, MI, USA), anti-β-actin (1:5000, Sigma–Aldrich, Milan, Italy) overnight at RT; (3) and with anti-rabbit and anti-mouse HRP conjugated secondary antibodies (1:10 000, Santa Cruz Biotechnology Inc., Dallas, USA) for 1 h. Blots were washed after each step. Then, chemiluminescent reagents (Immobilon, Millipore, MA, USA) were used to visualize immunoreactive bands with Kodak Image Station 440 (Eastman Kodak Company, Rochester, New York) and perform densitometric analysis. To ensure equal loading and accuracy of changes in protein expression, protein levels were normalized to ß-actin.

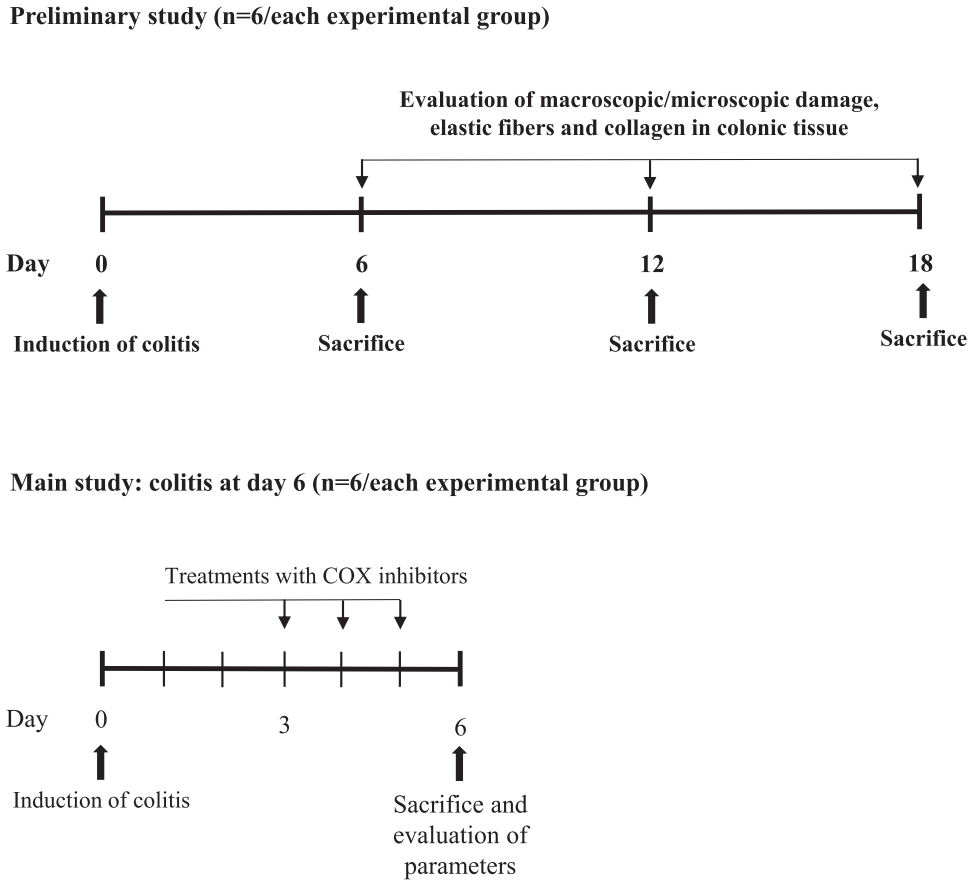

Figure 1 Timeline with details of the experimental design.

Table 1 Criteria for macroscopic scoring of colonic ulceration and inflammation

Score	*Appearance*
0	Normal
1	Localized hyperemia, no ulcers
2	Ulceration without hyperemia or bowel wall thickening
3	Ulceration with hyperemia at one site
4	Two or more sites of ulceration and hyperemia
5	Major sites of damage extending >1 cm along the length of colon
6	Area of damage extending >2 cm along the length of colon

All parameters of macroscopic damages were recorded and scored for each rat by two blinded to the treatment observers. Score was increased by 1 for each millimetreof bowel wall thickness. The presence of adhesions between colonic tissue and other organs (0, none; 1, minor; 2, major adhesions) and the consistency of colonic fecal material (0, formed; 1, loose; 2, liquid stools) were also scored.

Real-time polymerase chain reaction (PCR) analysis

Total mRNA from colonic samples was isolated with Qiagen kit and was transcribed into cDNA by One Step SYBR Prime Script real-time (RT) polymerase chain reaction (PCR) (Takara-Clontech, Kusatsu, Japan). The mRNA expression levels of collagen I and TGF-β were determined by RT-PCR using a relative quantification method with GAPDH as an endogenous control. GAPDH, Collagen I and TGF-β primers were purchased from Sigma–Aldrich Gene Expression Assay service, and were as follows: GAPDH 5′-ACATCAAGAAGGTGGTGA (F), 5′-GTCAAA GGTGGAGGAGTG (R); collagen I, 5′-GGA GAGTACTGGATCGACCCTAAC (F), antisense 5′-CTG ACCTGTCTCCATGTTGCA-3′ (R); TGF-β, 5′-GCCA CTGCCGGACAACTC-3′ (F), 5′-GCCGTGGATACTTGG AGTGACT-3′ (R).[19] Analyses were performed in 10 µl reaction volume in 48-well plates (Eco Real-Time PCR 48-well plate). Samples were run on Eco Real-Time PCR System (Illumina, Inc, San Diego, CA) and underwent PCR amplification for 30 cycles (1 min at 94°C, 1 min at 54°C, 1 min at 72°C). The threshold was set to the geometric phase of the amplification curve. Amplification products were normalized to GAPDH, and the quantification of gene expression was calculated using the formula $2\text{-}\Delta\Delta Ct$.

Recording of colonic peristaltic activity

A modified Trendelenburg set-up was used to elicit peristalsis activity in isolated colonic segments.[20] Distal colonic segments (approximately 6.5 cm) were mounted horizontally in organ bath containing 40 mL of Krebs solution (113 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 11.5 mM glucose, pH 7.4). The aboral end of colonic segment was secured to an open, adjustable outlet that could be raised in height.[21] The oral side of colonic segment was connected to a pressure transducer for recording changes in intraluminal pressure and to a perfusion pump maintaining continuous intraluminal infusion of warm carbogenated Krebs solution at a rate of 0.5

mL/min. Colonic segments were equilibrated for 30 min and the outlet was then progressively moved up to a height of 7.5 cm by increments of 2.5 cm every 20 min. This gradual distension of the colonic wall triggered rhythmic and repetitive peristaltic contractions propagating aborally, which were recorded by the pressure transducer connected at the oral side of the segment as cyclic pressure waves. In accordance with other studies,[20–22] the following parameters were evaluated: duration of the preparatory phase, volume of medium infused during the preparatory phase, ΔP threshold pressure; maximal ejection pressure.

Drugs

DNBS, indomethacin and celecoxib were purchased from Sigma Chemical, St Louis, Missouri, USA and SC-560 from Tocris, Bristol, UK.

Statistical analysis

Non-parametric test of Kruskal–Wallis, followed by Dunn's test for post-hoc group comparisons, was carried out since the study variables were not distributed normally. Histograms in graphs express data as mean ± standard deviation and they were considered significantly different when P values <0.05. All statistical procedures were performed using GraphPad Prism software (version 7.0, San Diego, CA, USA).

Results

Macroscopic and histological evaluation of colonic tissues

The macroscopic appearance of distal colon revealed significant bowel dilation, adhesions, ulcerations, wall thickening as compared with controls after 6 days colitis induction ($P < 0.0001$), and persisted over time (Supplementary Figure 1A). In addition, DNBS-treated rats displayed body weight loss (data not shown), diarrhea and hematochezia.

The histopathological damage was characterized by a significant loss of mucosal architecture and muscle thickening *versus* controls at days 6 ($P < 0.0001$). A partial recovery of microscopic damage scores was observed at the subsequent time points (day 12, $P = 0.007$; day 18, $P = 0.024$ Supplementary Figure 1B).

Evaluation of colonic fibrotic changes

The distribution pattern of colonic collagen (Figure 2A–D) and elastic fibres (Figure 2E–H) changed considerably in animals with colitis, as compared to controls. Indeed, colitic rats showed a significantly increased collagen deposition within the overall colonic wall, with abundant bundles of fibres in the *tunica submucosa* and *muscularis* at days 6 ($P = 0.018$), 12 ($P = 0.013$) and 18 ($P = 0.001$) *versus* the control group (Figure 2I). The elastic fibres, which were constitutively detected within vessel walls and along the myenteric ridge, displayed a significant decrease and disarrangement in animals with colitis at day 6 ($P < 0.0001$) and day 12 ($P = 0.030$) as compared with the control group, with a subsequent progressive recovery up to normalized levels and distribution patterns versus day 6 at day 18 ($P < 0.0001$; Figure 2J).

After 3 days of treatments with indomethacin, SC-560 and celecoxib in animals with colitis was induced a significant decrease in colonic collagen deposition in the whole-thickness wall, as compared to animals with colitis treated with vehicle ($P = 0.042$, $P = 0.004$, $P = 0.010$, respectively; Figure 3A). The hydroxyproline content, that climbed up to significant levels in colonic samples from inflamed animals as compared to controls ($P = 0.001$), was counteracted by treatment with indomethacin ($P = 0.042$) and celecoxib ($P = 0.017$) (Figure 3B). The elastic fibres, which decreased significantly in animals with colitis at day 6, drifted towards a recovery after treatment with SC-560 ($P = 0.023$) and celecoxib ($P = 0.020$) (Figure 3C).

Analysis of molecular factors related with fibrosis

Collagen I and III, fibronectin, MMP-2, MMP-9, COX-1 and COX-2 expression were increased in colonic tissues after 6 days colitis induction, when compared to normal animals (Figure 4). The significant increments of collagen I ($P = 0.008$), collagen III ($P < 0.0001$) and fibronectin ($P < 0.0001$) expression, as observed in DNBS-animals, were reverted back towards normal levels in animals treated with COX inhibitors, with the following statistical values: indomethacin ($P = 0.047$), SC-560 ($P = 0.012$) and celecoxib ($P = 0.014$) for collagen I; indomethacin ($P = 0.006$) for collagen III; indomethacin ($P = 0.009$) for fibronectin (Figure 5A and B). Collagen I mRNA levels and protein expression displayed a similar trend (Figure 5C) in all treatment groups ($P = 0.0001$).

Recording of colonic peristaltic activity

The distension of distal colonic segments resulted in repetitive and reproducible peristaltic pressure waves. Representative tracings of peristaltic activity in the colon from control and DNBS animals are shown in Figure 6A. Animals with colitis displayed an impairment of colonic peristalsis, as demonstrated by a significant decrease in maximal ejection pressure ($P < 0.0001$) as well as an increase in the threshold pressure required to trigger peristalsis ($P < 0.0001$), the duration of preparatory phase ($P < 0.0001$) and the volume in the preparatory phase ($P = 0.002$). These functional parameters were restored back to the values recorded in normal animals by the COX inhibitors, with the following statistical results: indomethacin ($P = 0.031$) for ejection; SC560 ($P = 0.036$) for threshold; indomethacin ($P = 0.020$) for duration; indomethacin ($P = 0.027$) and celecoxib ($P = 0.018$) for volume (Figure 6B).

Analysis of TGF-β and related molecular factors

The effects of COX inhibitors were tested on the colonic expression of TGF-β signalling cascade, such as TGF-β itself, RhoA, phosphorylated SMAD2 (pSMAD2) and SMAD6, both in control and inflamed animals. Under the present experimental conditions, TGF-β ($P = 0.004$), RhoA ($P < 0.0001$) and pSMAD2 ($P < 0.0001$) expression were significantly upregulated in the colon from inflamed animals, when compared to normal animals (Figure 7A and B). These changes were counteracted by treatments with indomethacin, SC-560 and celecoxib with the following statistical values: SC-560 ($P = 0.021$) and celecoxib ($P = 0.001$) for TGF-β, SC-560 ($P = 0.048$) and celecoxib ($P = 0.005$) for RhoA, celecoxib ($P = 0.006$) for SMAD2. On the other hand, colonic SMAD6 levels were unchanged in animals with colitis,

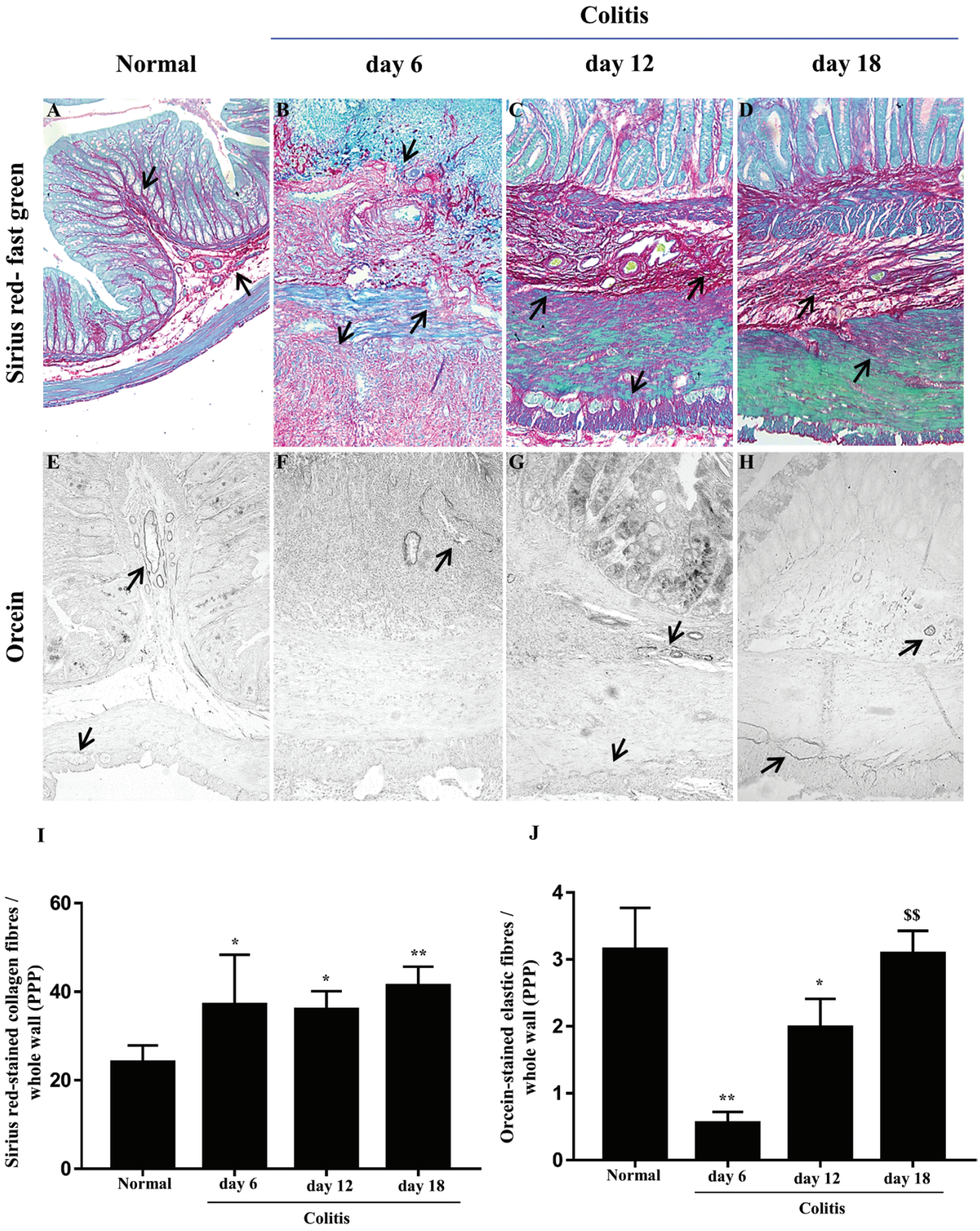

Figure 2 Histological assessment of colonic collagen and elastic fibres at day 6, 12 and 18 after the induction of colitis. Representative Sirius red-fast green (A–D) and orcein (E–H) images of collagen fibres (red-stained, arrows) and elastic fibres (black-stained, arrows), detected in the whole-thickness distal colon from normal and inflamed rats on days 6, 12 and 18 (n = 6/group), with respective quantitative evaluations (I and J). Column graphs display the mean values of whole-thickness positive pixels percentage (PPP) ± standard deviation (SD) obtained from 6 animals for each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus normal rats; $^{\$\$}P < 0.01$ versus colitis rats at day 6. Original magnification: 10×.

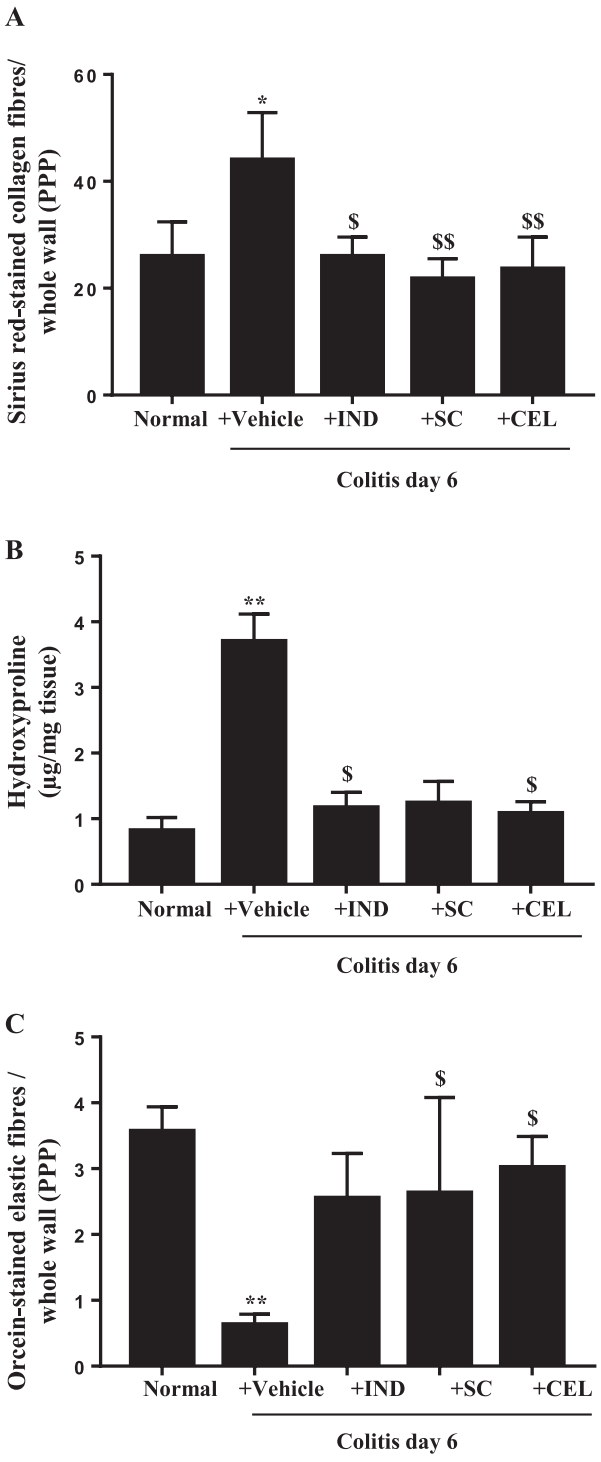

Figure 3 Effects of indomethacin (IND), SC-560 (SC) and celecoxib (CEL) on colonic collagen and elastic fibres at day 6 after the induction of colitis. Quantitative estimations of Sirius red-stained collagen (A) and orcein-stained elastic (C) fibres in the whole-thickness wall of colonic samples (n = 6/group). Quantification of hydroxyproline levels in colonic full-thickness samples (B). Column graphs display the mean values of whole-thickness positive pixels percentage (PPP) or hydroxyproline content/tissue (µg/mg) ± standard deviation (SD) obtained from 6 animals for each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus normal rats; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ versus vehicle-treated colitis rats.

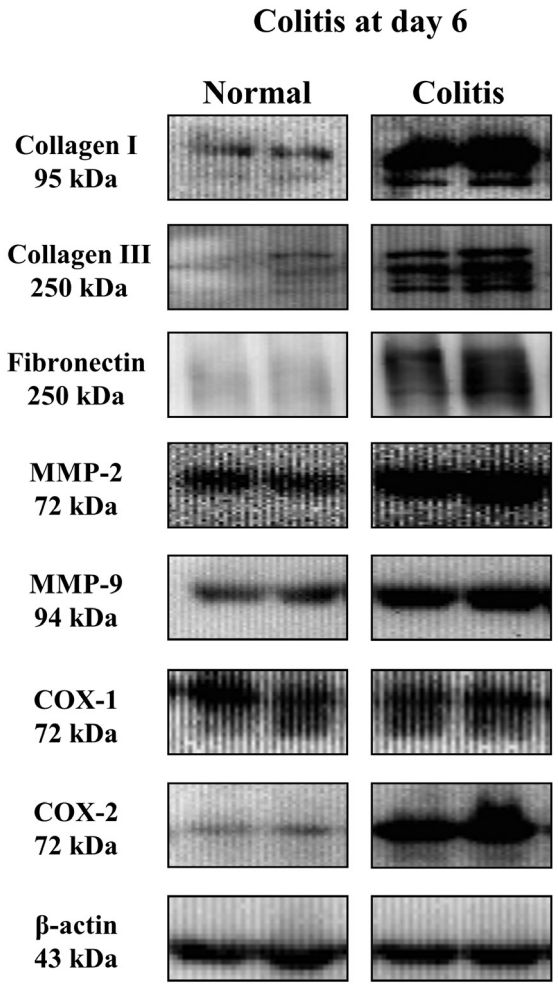

Figure 4 Western blot analysis of the colonic expression of molecular factors related with fibrosis and cyclooxygenase isoforms. Representative blots showing the expression of collagen I, collagen III, fibronectin, type 2 matrix metalloproteinase (MMP-2), type 9 MMP (MMP-9), cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) in normal controls and rats with colitis at day 6 (n = 6/group).

and such an expression pattern was upregulated by treatments with COX inhibitors with the following values: SC-560 (P = 0.014) and celecoxib (P = 0.001) (Figure 7A and B). A similar trend of TGF-β mRNA levels was detected in PCR analysis (Figure 7C).

Discussion

Fibrosis is a chronic and progressive pathological process, characterized by an excessive deposition of extracellular matrix components, such as collagen, which usually follows and accompanies a chronic inflammatory condition. Such a process results from reiterated, yet abnormal, efforts of achieving healing and repair in chronically inflamed tissues, and it is known to affect almost all organs.[23]

Several evidences point out that COX pathways are involved in fibrogenic processes of several body districts. Indeed, COX-2 inhibitors, including celecoxib, ameliorated hepatic damage and decreased intra-hepatic collagen 1 deposition in rat liver fibrosis, exerting antifibrotic/fibrolytic effects.[4, 5] COX-2 inhibitors also blocked the fibrogenic response of activated hepatic stellate cells and tissue TGF-β

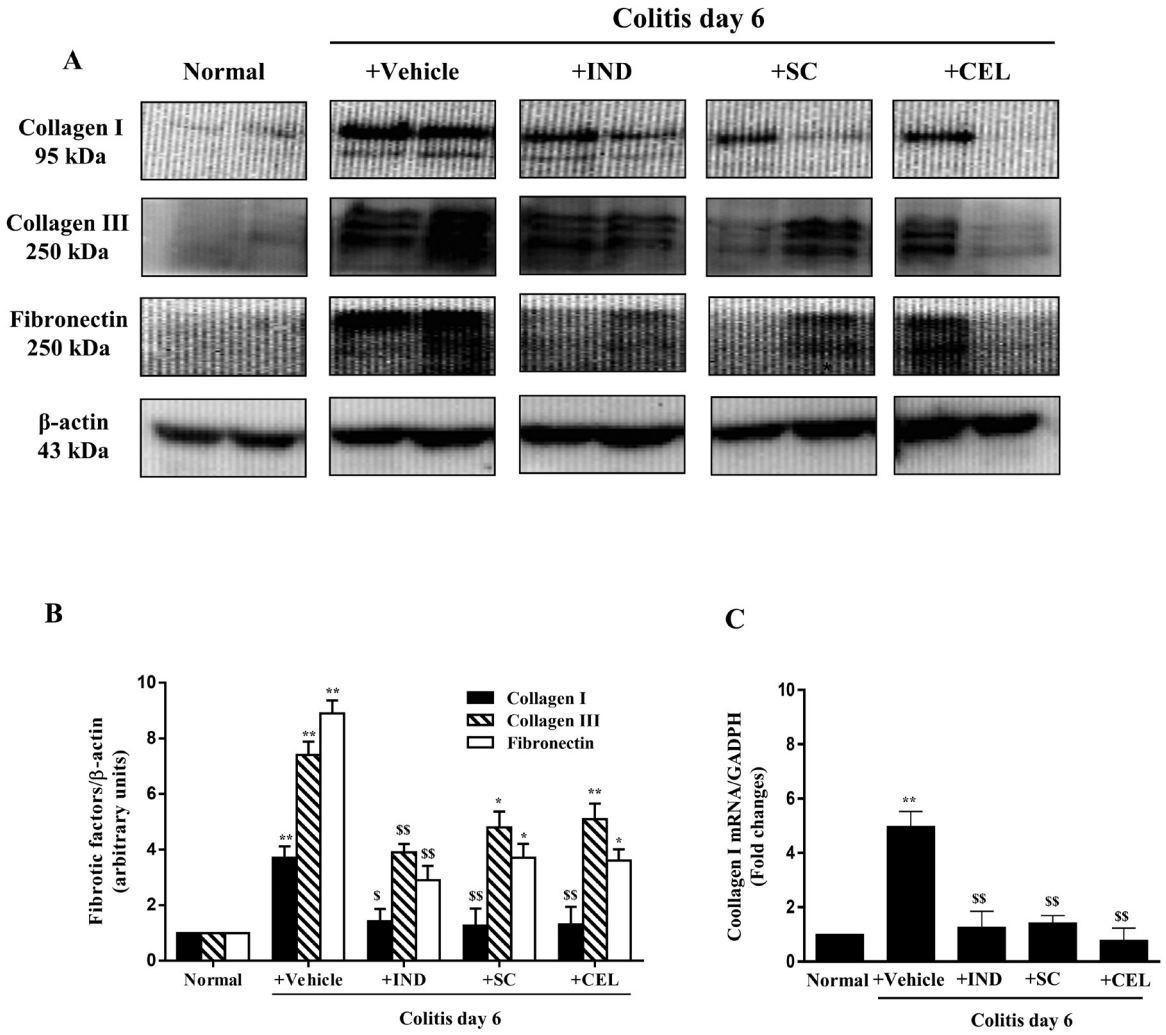

Figure 5 Effects of cyclooxygenase inhibitors on molecular factors related to fibrosis. (A) Western blot analysis of the colonic expression of collagen I, collagen III and fibronectin in normal controls and animals with colitis at day 6, in the absence or in the presence of treatments with indomethacin (IND), SC-560 (SC) or celecoxib (CEL). (B) Quantitative assessments of immunopositive bands by densitometric analysis (n = 6/group). (C) PCR analysis of collagen I mRNA expression in colonic samples in the respective experimental groups. Each column represents the mean ± standard deviation (SD) obtained from 6 animals. $^{**}P < 0.01$ versus normal rats; $^{\$\$}P < 0.01$ versus vehicle-treated colitis rats.

and collagen I mRNA in different hepatic fibrosis models.[24] COX-2 inhibitors have been shown to reduce the number of fibroblasts and rate of collagen biosynthesis in *Salmonella*-induced enterocolitis.[25] Moreover, a previous study showed that COX inhibition exerted beneficial effects in DNBS-induced colitis.[26] Indeed, tough COX-derived PGE_2 has been found to be involved in the maintenance of gut epithelium,[10–12] a recent study reported the role of PGE_2 in shaping immune/inflammatory responses during inflammation through EP4 receptors.[13]

Based on the above background, in this study, we examined the effects of both selective and non-selective COX inhibitors in an experimental model of intestinal fibrosis associated with colitis, and the following main findings were highlighted: (1) a reduction of collagen and fibronectin deposition along with a restoration of elastic fibres; (2) a decrease in the expression of TGF-β, RhoA and pSMAD2, and an increased expression of the inhibitory factor SMAD6; (3) a prevention of colonic propulsive motor alterations.

Before testing the effects of COX inhibitors, the experimental model employed in this study was investigated for the presence of fibrosis, associated with the induction of colitis. The DNBS-induced colitis is characterized by an increase in tissue and circulating inflammatory biomarkers, including tumour necrosis factor, interleukin-1beta (IL-1β), IL-6 and PGE_2.[26–29] Colonic inflammation contributes to the occurrence of fibrotic processes and, indeed, we observed that, after 6 days colitis induction, the colonic wall displayed a significant degree of fibrotic remodelling. In particular, an increase in collagen deposition along with decreased elastic fibres in the colonic wall was detected in animals with colitis. Therefore, treatments with COX-1 (SC-560), COX-2 (celecoxib) or COX-1/COX-2 (indomethacin) inhibitors started after 3 days from DNBS injection and continued until the time of sacrifice, with the aim of investigating the molecular events involved in fibrogenesis.

Our results show that the COX-1 and COX-2 inhibition pathways in rats with colitis exerted inhibitory effects on colonic fibrosis, through a decrease in tissue matrix components expression (collagen I and III, fibronectin) and pro-fibrotic factors (TGF-β, RhoA and pSMAD2), suggesting that both COX-1 and COX-2 pathways contribute to the pathophysiology of

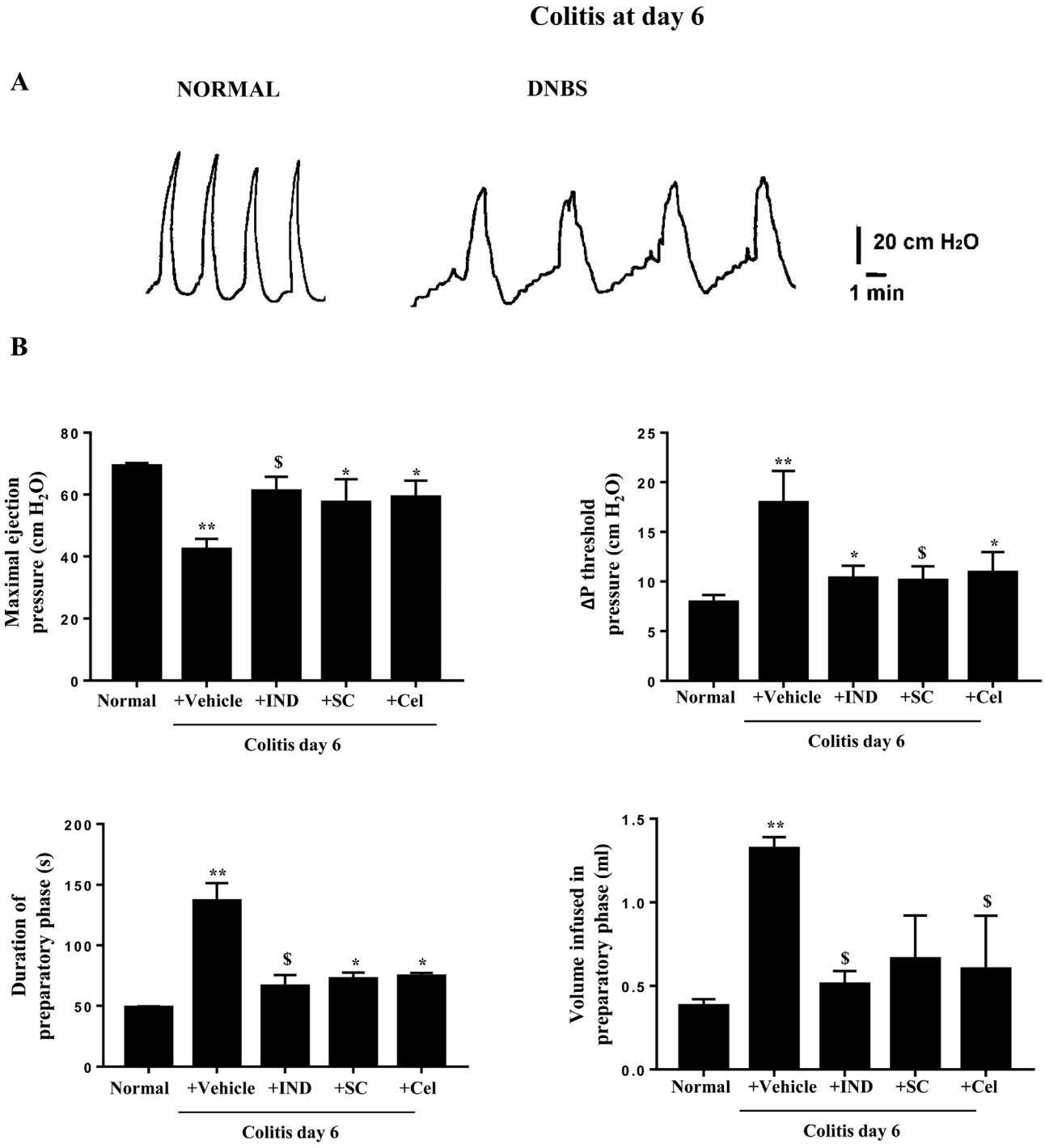

Figure 6 Effects of cyclooxygenase inhibitors on in-vitro colonic peristaltic activity. (A) Representative tracings of peristaltic waves induced by intraluminal pressure increments in preparations of distal colon from normal controls and animals with colitis at day 6. (B) Effects of indomethacin (IND, 2 mg/kg), SC-560 (SC, 2.5 mg/kg) and celecoxib (CEL, 1 mg/kg) on colonic peristaltic activity in animals with colitis (n = 6/group). Each column represents the mean ± standard deviation (SD) obtained from 6 animals. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus normal rats; $^{\$}P < 0.05$ versus vehicle-treated colitis rats.

colonic fibrotic remodelling. In line with our results, several studies have shown that both COX-1 and COX-2 are involved in shaping immune/inflammatory responses during inflammation.[30, 31] For instance, PGE_2 has been shown to contribute to the progression of acute and chronic inflammation and autoimmune diseases.[30] In these conditions, the pharmacological blockade of COX-1, COX-2 or COX-1/2 can counteract immune/inflammatory responses.[30]

When considering the regulatory role played by COX pathways in fibrotic remodelling, it is noteworthy that prostaglandins, generated by COX pathways, can induce the expression of TGF-β,[32, 33] regarded as the main effector of tissue fibrogenesis. The pro-fibrotic actions of TGF-β are driven by SMAD2 and SMAD3 activation, which undergo phosphorylation when TGF-β binds its own receptors.[34, 35] Indeed, COX inhibition blocked the activation of the TGF-β/

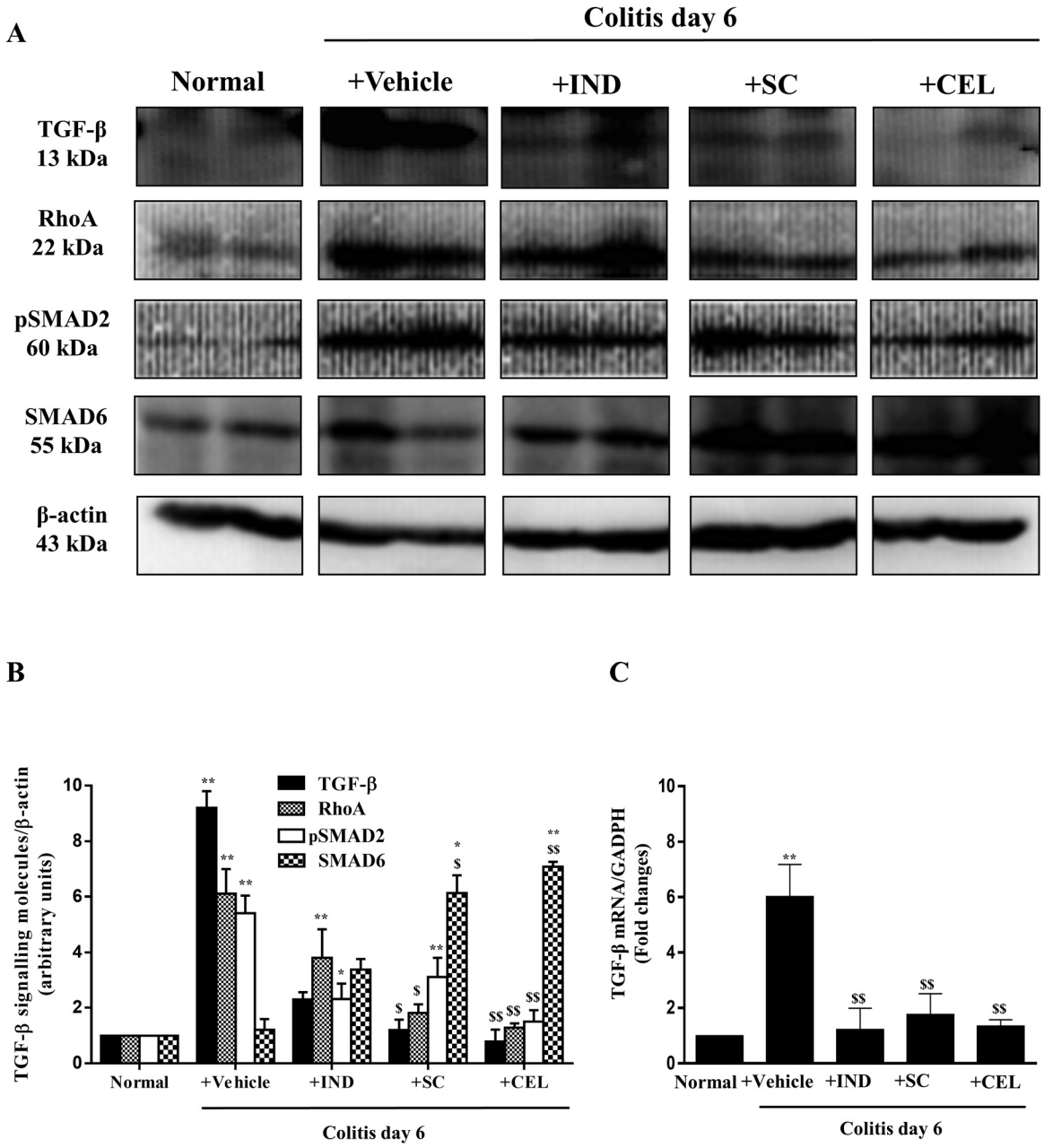

Figure 7 Effects of COX inhibitors on molecular factors related to TGF-β signalling. (A) Western blot analysis of the expression of TGF-β, RhoA, phospho-SMAD2 (pSMAD2) and SMAD6 in colonic specimens from normal controls and animals with colitis at day 6, in the absence and in the presence of treatments with indomethacin (IND), SC-560 (SC) and celecoxib (CEL). (B) Quantitative assessments of immunopositive bands by densitometric analysis (n = 6/group). (C) PCR analysis of TGF-β mRNA expression in colonic samples of the respective experimental groups. Each column represents the mean ± standard deviation (SD) obtained from 6 animals. $^{**}P < 0.01$ versus normal rats; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ versus vehicle-treated colitis rats.

SMAD2/SMAD3 pathway in glomerular mesangial cells.[36] In this study, there was an upregulation of TGF-β and phospho-SMAD2 in the inflamed colon, and COX inhibitors reduced both TGF-β and phospho-SMAD2 expression, confirming the anti-fibrotic effects following COX inhibition.

Of note, the pro-fibrotic activity of the TGF-β/SMAD2 pathway can be downregulated by the induction of inhibitory, anti-fibrotic factors, such as SMAD6 and SMAD7.[37] In this respect, we observed that COX inhibitors increased SMAD6 expression in colonic tissues, and such an effect likely contributed to counteracting the enteric pro-fibrotic activity mediated by endogenous TGF-β.

According to current literature, an additional fibrogenic signal activated by TGF-β is represented by the RhoA pathway.[35, 38] Indeed, there is also evidence involving RhoA signalling in the pathophysiology of bowel fibrosis.[38, 39] Of note, COX inhibitors, including ibuprofen, indomethacin and naproxen, inhibited RhoA signalling in cultured neurons.[40, 41] This body of data prompted us to investigate the effects of COX inhibitors on RhoA in our model, and, we found that RhoA expression underwent upregulation in fibrotic colon, and such an increment was significantly counteracted by all tested COX inhibitors. These results support the view that, besides interfering with SMAD2 signalling, the pharmacological

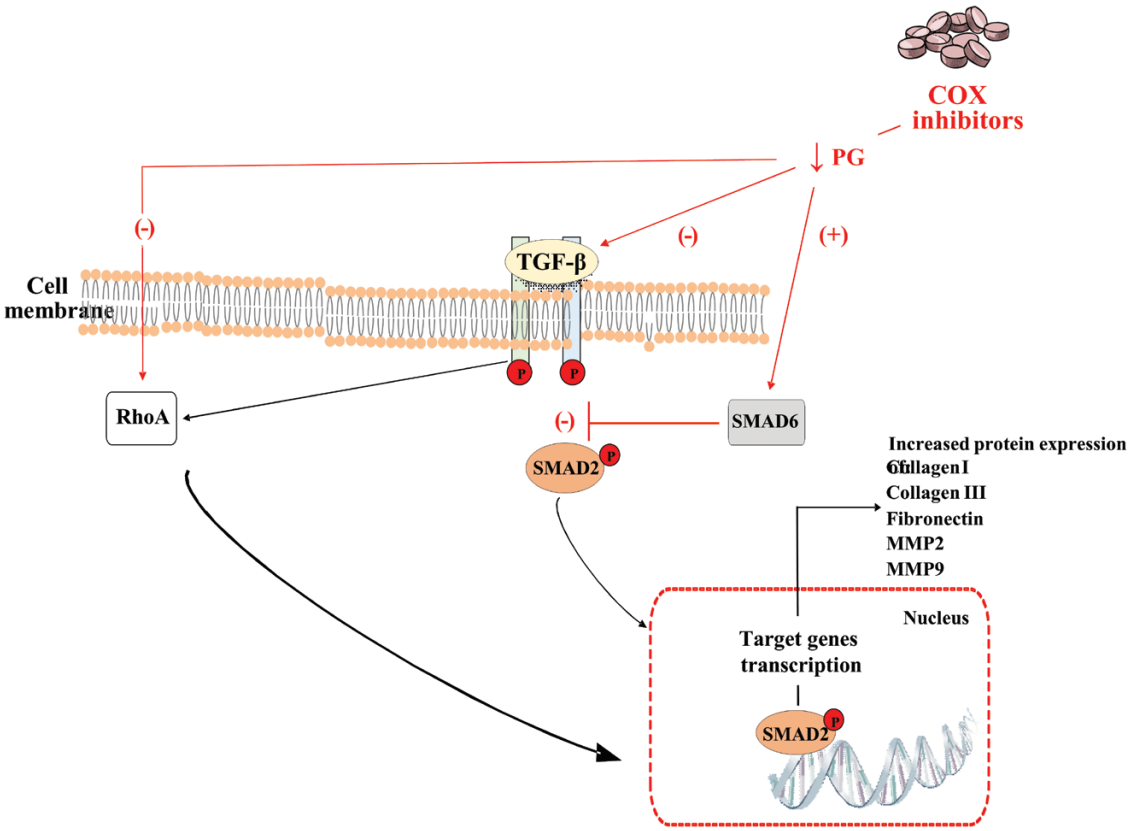

Figure 8 Schematic diagram illustrating TGF-β signalling pathways involved in intestinal fibrosis and the possible molecular factors targeted by cyclooxygenase inhibitors.

blockade of COX pathways could hamper also the fibrogenic activity of endogenous TGF-β via downregulation of RhoA signalling. The mechanisms underlying such effects, besides the decrease in PG production, could be also depend on COX-independent effects.[42–44]

It is widely acknowledged that an excess of collagenic matrix deposition in hollow organs results in a pathological increment of their wall stiffness, with subsequent alterations of their neuromuscular functions.[45-47] Such motor dysfunctions can contribute to derangements of enteric propulsive motility[48] For these reasons, we evaluated the impact of fibrotic changes on peristaltic motility elicited by intraluminal pressure increments in isolated colonic segments. Indeed, peristalsis is a highly integrated and coordinated pattern of autonomous motor activity that ensures the propulsion of its luminal contents throughout the different phases of its physiological tasks. Any factor interfering with triggering and performance of the peristaltic reflex can impair the implementation of propulsive motility.[49] In our hands, the onset and propagation of peristaltic motor activity in colonic preparations from animals with colitis were significantly altered. When recording the motor activity of fibrotic colonic segments from animals treated with COX inhibitors, we observed a normalization of the patterns of peristaltic contractions. Of note, COX inhibitors were able to normalize almost all the four parameters recorded to estimate quantitatively the performance of peristaltic activity. This finding is in line with our previous results showing that COX pathways are significantly involved in the control of digestive motility.[50, 51] However, the anti-fibrotic effect of COX inhibitors, action appears to contribute to the recovery of peristaltic activity. In support of this contention, our experiments demonstrated that COX inhibitors improved the threshold pressure and the volume of medium infused over the preparatory phase, with consequent improvement of peristaltic motility.[52]

Conclusions

This study shows that inflammation-related gut fibrosis is associated with impairment of bowel propulsive motility and activation of COX and TGF-β pathways. In this setting, the pharmacological inhibition of COX-1 and COX-2 can counteract colonic fibrotic remodelling through downregulation of TGF-β and related SMAD/RhoA signalling by decreasing prostanoid production (Figure 8). Even though NSAIDs do not hold a role in the therapy of IBDs, these findings open and may encourage additional investigations on the search of novel antifibrotic therapeutic interventions based on targeting of COX and/or TGF-β pathways.

Supplementary Material

Supplementary data are available at *Journal of Pharmacy and Pharmacology* online.

Author Contributions

R. Colucci, M. Fornai, L. Antonioli and C. Blandizzi designed research; C. Pellegrini, C. Ippolito, C. Segnani analysed

data; R. Colucci, M. Fornai, L. Antonioli, M.G. Zizzo, C. Ippolito, C. Segnani, N. Bernardini, A. Nericcio performed research; R. Colucci, N. Bernardini, R. Serio and C. Blandizzi wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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