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The cyclo-oxygenase-2 inhibitor, rofecoxib, attenuates mucosal damage due to colitis induced by trinitrobenzene sulphonic acid in rats

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Abstract

Cyclo-oxygenase-2 overexpression has been described in experimental colitis. However, there are controversial findings suggesting that its inhibition by selective cyclo-oxygenase-2 inhibitors not only may have a beneficial effect on experimental colitis, but also exacerbate the inflammation-associated colonic injury. Thus, the role of cyclo-oxygenase-2 inhibitors in the possible modulation of colon inflammation is controversial and remains uncertain. In this study, we evaluated the effects of the selective cyclo-oxygenase-2 inhibitor, rofecoxib, on the extent and severity of ulcerative colitis caused by intracolonic administration of 2,4,6-trinitrobenzene sulphonic acid (TNBS) in rats. The lesions and the inflammatory response were assessed by histology and measurement of myeloperoxidase activity. Interleukin-1β, prostaglandin E_2 and D_2 levels in colon mucosa and the immunohistochemical expression of the cyclo-oxygenases-1 and -2 were also studied. Finally, we investigated the effects of rofecoxib on apoptosis of colonocytes by the appearance of DNA fragmentation. Inflammation following TNBS was characterized by increased colonic wall thickness, oedema, diffuse inflammatory cell infiltration in the mucosa, and necrosis. Increased myeloperoxidase activity, as an index of neutrophil infiltration in the mucosa, and interleukin-1β levels were also measured in the colon. Administration of rofecoxib significantly ($P<0.05$) reduced the colonic damage, the degree of neutrophil infiltration, and interleukin-1β levels. In addition, apoptosis was significantly increased in TNBS-treated rats, but not in rofecoxib plus TNBS-treated rats. We concluded that rofecoxib seems to have beneficial effects in TNBS-induced colitis by diminishing the initial stage of inflammation, probably by a mechanism related to inhibition of prostaglandin E_2 by the cyclo-oxygenase-2 pathway. The data suggest that cyclo-oxygenase-2-selective inhibitors may have a therapeutic role in ulcerative colitis.

Keywords: Cyclo-oxygenase-2; Colitis; TNBS (trinitrobenzene sulphonic acid); Neutrophil; Interleukin-1β; Prostaglandin; Apoptosis

1. Introduction

Inflammatory bowel disease, which includes ulcerative colitis and Crohn's disease, is a chronic relapsing and nonspecific inflammatory disorder resulting from the transmural infiltration of neutrophils, macrophages, lymphocytes, and mast cells, ultimately giving rise to mucosal disruption and ulceration (Fiocchi, 1998). The infiltrated and activated neutrophils represent an important source of reactive oxygen and nitrogen species. These species are cytotoxic agents, inducing cellular oxidative stress by cross-linking proteins, lipids, and nucleic acids, causing cellular dysfunction and damage. In addition to free radicals, neutrophils can also release proteases, lactoferrin and lipid mediators that can contribute to intestinal injury. Macrophages produce certain cytokines, such as tumor necrosis factor (TNF-α) and interleukin-1β, the levels of which are often increased in both animal models and patients with ulcerative colitis (Rogler and Andus, 1998; Nitta et al., 2002). In addition, interleukin-1β appears to be a primary stimulator of diarrhoea, the major symptom of intestinal inflammation, and is cleaved and activated by the interleukin-1β-converting enzyme (Siegmund, 2002).

Nonsteroidal anti-inflammatory drugs (NSAID) are widely used, but their use in the treatment of pain, fever and inflammation is associated with significant untoward effects on the gastrointestinal tract caused mainly by the inhibition of the synthesis of prostaglandins via cyclo-oxygenase enzymes. Two isoforms of cyclo-oxygenase are

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recognized: cyclo-oxygenase-1 is a constitutively expressed enzyme in many tissues, including gastrointestinal tract, while cyclo-oxygenase-2 is an inducible enzyme predominantly expressed at sites of inflammation (Simon, 1998; Vane and Botting, 1998; Warner et al., 1999). There is evidence that prostanoids produced via cyclo-oxygenase-2 contribute to inflammation, pain, and fever, and that prostanoids produced by cyclo-oxygenase-1 exert immunomodulatory, cytoprotective and proangiogenic effects (Dannhart and Kiefer, 2001). Moreover, cyclo-oxygenase-2 produces prostaglandins that inhibit apoptosis and promote early colon carcinogenesis (Gupta and Dubois, 2001). In inflammatory bowel disease and experimental colitis, increased amounts of cyclo-oxygenase-2 mRNA and protein have been found in inflamed areas, and most of the prostaglandins are produced by the cyclo-oxygenase-2 pathway (Singer et al., 1998). Thus, cyclo-oxygenase-2 inhibitors would be useful in the prevention of colorectal carcinomas that arise in the setting of inflammatory bowel disease (Agoff et al., 2000).

It has been recently demonstrated that some cyclo-oxygenase-2 inhibitors do not have a beneficial effect in an experimental model of colitis (Lesch et al., 1999) and exacerbate inflammation-associated colonic injury (Reuter et al., 1996). In contrast, others investigators (Kankuri et al., 2001; Karmeli et al., 2000; Cuzzocrea et al., 2001; Campbell et al., 2002; Khan et al., 2002) demonstrated that cyclo-oxygenase-2 inhibitors had a beneficial effect on experimental colitis in rats, acting by reducing colonic eicosanoid generation and the associated oxidative stress.

The effects of cyclo-oxygenase-2 inhibitor therapy in patients with inflammatory bowel disease have been evaluated recently (Bonner et al., 2000; Bonner, 2002). This study failed to demonstrate a correlation between NSAIDs use and likelihood of active inflammatory bowel disease. In addition, Mahadevan et al. (2002) reported the safety and beneficial effects of cyclo-oxygenase-2 inhibitors in patients with inflammatory bowel disease. Thus, the role of cyclo-oxygenase-2 inhibitors in the possible modulation of colon inflammation is controversial and remains uncertain. These data prompted us to study the effects of rofecoxib, a new cyclo-oxygenase-2 inhibitor, on acute experimental trinitrobenzene sulphonic acid (TNBS)-induced colitis in rats. We determined the production of prostaglandin E_2 and D_2 in colon mucosa and the expression of cyclo-oxygenases-1 and -2 inmunohistochemically.

As mentioned above, oxygen free radicals, neutrophils, and proinflammatory cytokines are clearly involved in the pathogenesis of inflammatory bowel disease. Accordingly, the inflammatory response was assessed by histology and by measuring myeloperoxidase activity as an index of quantitative inflammation and neutrophil infiltration in the mucosa. Mucosal interleukin-1β production was measured and histological and histochemical analysis of the lesions was also carried out. Finally, since cyclo-oxygenase-2 inhibitors have been found to modulate apoptosis (Yamazaki et al., 2002), we investigated their effects on colonic mucosa during an early acute phase of inflammation.

2. Materials and methods

2.1. Experimental animals

Male Wistar rats supplied by Animal Services, Faculty of Medicine, University of Seville, Spain, and weighing 180–220 g, were placed in individual cages with wire-net floors in a controlled room (temperature 24–25 °C, humidity 70–75%, lighting regimen of 12L/12D) and were fed on a normal laboratory diet (Panlab, Barcelona, Spain). Rats were deprived of food for 24 h prior to the induction of colitis, but were allowed free access to tap water throughout. They were randomly assigned to groups of 8–14 animals. Experiments followed a protocol approved by the local animal Ethics Committee and the Local Government. All experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Counsel 86/609/EC).

2.2. Induction of colitis

Colitis was induced according to the procedure described by Morris et al. (1989). Briefly, rats were lightly anesthetized with ether following a 24-h fast, and then a medical-grade polyurethane cannula for enteral feeding (external diameter 2 mm) was inserted into the anus and the tip was advanced to 8 cm proximal to the anal verge. TNBS (Sigma, Spain) dissolved in 50% ethanol was instilled into the colon through the cannula (10 mg in a volume of 0.25 ml to induce acute colitis). Following the instillation of the hapten, the animals were maintained in a head-down position for a few minutes to prevent leakage of the intracolonic instillate. Different control groups were created for comparison with TNBS/ethanol instillation: rats in the normal group received an enema of physiological saline instead of the TNBS solution, and rats in the ethanol control group received 0.25 ml 50% ethanol. Rofecoxib (1.25, 2.5 and 5 mg/kg; Merck Sharp and Dohme, Spain) was suspended in 0.9% saline solution and administered (p.o.) 48, 24 and 1 h prior to the induction of colitis and 24 h later. Control groups received vehicle in a comparable volume (10 ml/kg body weight). The animals were killed, using an overdose of anesthetic, 48 h after induction of colitis.

The rats were checked daily for behaviour, body weight, and stool consistency.

2.3. Assessment of colitis

The severity of colitis was evaluated by an independent observer who was blinded to the treatment. For each animal,

the distal 10-cm portion of the colon was removed and cut longitudinally, cleaned in physiological saline to remove faecal residue, and weighed. Macroscopic inflammation scores were assigned based on clinical features of the colon (score 0–10), the presence of adhesions between colon and small bowel and other organs (score 0–2), and/or stool consistency (score 0–1), according to the criteria of Bobin-Dubigeon et al. (2001). The colon was subsequently divided longitudinally into pieces and frozen in liquid nitrogen for measurement of myeloperoxidase activity, interleukin-1β levels and prostaglandin production.

2.4. Histological studies

For examination with the light microscope, we used tissue samples from the distal colon of each animal fixed in 4% buffered paraformaldehyde, dehydrated in grade ethanol, and embedded in paraffin. Thereafter, 7-μm sections of tissue were cut on a rotary microtome (Leica Ultracut), mounted on clean glass slides and dried overnight at 37 °C. Sections were cleared, hydrated, and stained with haematoxylin and eosin or with Alcian blue for histological evaluation of colonic damage and mucus content, respectively, according to standard protocols (Torres et al., 1999). The slides were coded to prevent observer bias during evaluation. All tissue sections were examined under an Olympus BH-2 microscope for characterization of histopathological changes.

Photographs taken from colon samples were digitized using Kodak D290 Zoom camera Eastman Kodak, USA and Motic®Images 2000 release 1.1, (MicroOptic Industrial Group B1 Series System Microscopes). Analysis of the figures was carried out by using Adobe®Photoshop® Version 5.

2.5. Immunohistochemical study

Colonic tissues were fixed in 4% buffered paraformaldehyde, dehydrated through graded concentrations of ethanol, embedded in paraffin, and sectioned. Sections (5 μm thick) were mounted on slides, cleared, and hydrated. They were treated with a buffered blocking solution (3% bovine serum albumin) for 15 min. Then, sections were co-incubated with primary antibodies for cyclo-oxygenase-1 and cyclo-oxygenase-2 (goat polyclonal, M-19 and M-20 of Santa Cruz Biotechnologies) at a dilution of 1:400 at room temperature for 1 and 24 h, respectively. Sections were washed with PBS and co-incubated with secondary antibody (anti-sheep immunoglobulin G (IgG), peroxidase conjugated, Sigma, Spain) (1:500 in phosphate-buffered saline (PBS), v/v) at room temperature for 1 h. Thereafter, sections were washed as before and with Tris–HCl 0.05 M, pH 7.66, and then co-incubated with 3,3′-diaminobencidine solution in the dark at room temperature for 10 min. Sections were washed with Tris–HCl, stained with haematoxylin according to standard protocols (Torres et al., 1999) and observed under an Olympus BH-2 microscope.

2.6. Assessment of leukocyte involvement

Myeloperoxidase activity was assessed as a marker of neutrophil (PMN) infiltration according to the methods of Grisham et al. (1990). In all animals one sample from the distal colon was obtained. Samples were excised from each animal and rapidly rinsed with ice-cold saline, blotted dry, and frozen at −70 °C. The tissue was thawed, weighed and homogenized in 10 volumes 50 mM PBS, pH = 7.4. The homogenate was centrifuged at 20,000 × *g*, 20 min, 4 °C. The pellet was again homogenized in 10 volumes of 50 mM PBS, pH = 6.0, containing 0.5% hexadecyl-trimethylammonium bromide (HETAB) and 10 mM ethylenediamine tetraacetic acid (EDTA). This homogenate was subjected to one cycle of freezing/thawing and a brief period of sonication. A sample of homogenate (0.5 μl) was added to a 0.5-ml reaction volume containing 80 mM PBS, pH 5.4, 0.5% HETAB and 1.6 mM 3,3′,5,5′-tetramethylbenzidine (TMB). The mixture was incubated at 37 °C for 5 min and the reaction was started by the addition of 0.3 mM H_2O_2.

Table 1
Parameters quantified after administration of rofecoxib (1.25, 2.5 and 5 mg/kg) in rats with acute colitis induced by TNBS intracolonic instillation (10 mg/animal)

Group	*n*	Macroscopic damage (score 0–10)	Body weight changes (g)	Food consumption (g/rat-day)	Adhesions (score 0–2)	Diarrhoea (score 0–1)	Colon weight/colon length (mg/cm)
Sham	11	0.0 (0)	16.0 ± 4.3	25.7	0 (0)	0 (0)	133 ± 6
EtOH	15	2.4 (0–5)	1.4 ± 2.9	27.5	0 (0)	0.2 (0–1)	201 ± 24
TNBS	14	7.2 (3–9)	− 8.3 ± 12.3	18.5	1.5 (0–2)[a,b]	0.7 (0–1)	262 ± 35[a]
R 1.25	12	2.4 (0–6)[a]	5.0 ± 13.8	21.9	0.6 (0–2)	0.2 (0–1)	194 ± 18
R 2.5	12	3.2 (0–6)[a]	2.3 ± 15.6	18.9	1.0 (0–2)	0.4 (0–1)	236 ± 15
R 5	12	2.8 (0–9)[a]	6.3 ± 16.4	21.4	0.5 (0–2)	0.5 (0–1)	232 ± 22

Colonic parameters were quantified in the sham group ($n = 5$), which received saline instillation. TNBS group ($n = 6$) received trinitrobenzene sulphonic acid intracolonically in a vehicle of 50% ethanol; ethanol group ($n = 6$) received 50% ethanol intracolonic injection. Data are expressed as means ± S.E.M.

[a] $P < 0.05$ significantly different from sham.

[b] $P < 0.05$ significantly different from EtOH.

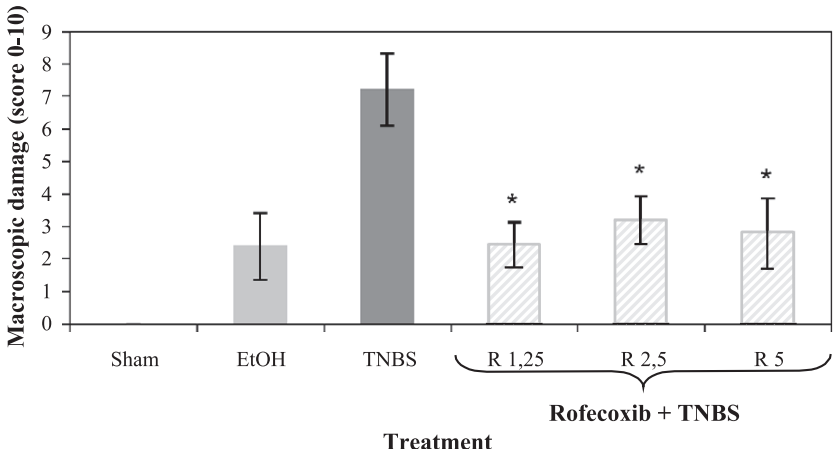

Fig. 1. Effects of acute administration of rofecoxib on the colonic damage score. Colonic macroscopic damage resulting from trinitrobenzene sulphonic acid (10 mg/animal) instilled into rat colon was scored as indicated in Materials and methods. Scores were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham group, trinitobenzene sulphonic acid group and ethanol group), or in the presence of rofecoxib (1.25, 2.5 and 5 mg/kg/day). Data are expressed as means ± S.E.M. (*$P < 0.05$ vs. TNBS group).

Each tube containing the complete reaction mixture was incubated for exactly 3 min at 37 °C. The reaction was terminated by the sequential addition of catalase (20 μg/ml) and 2 ml 0.2 M sodium acetate, pH = 3.0. The changes in absorbance at 655 nm were measured with a spectrophotometer. One unit of myeloperoxidase activity was defined

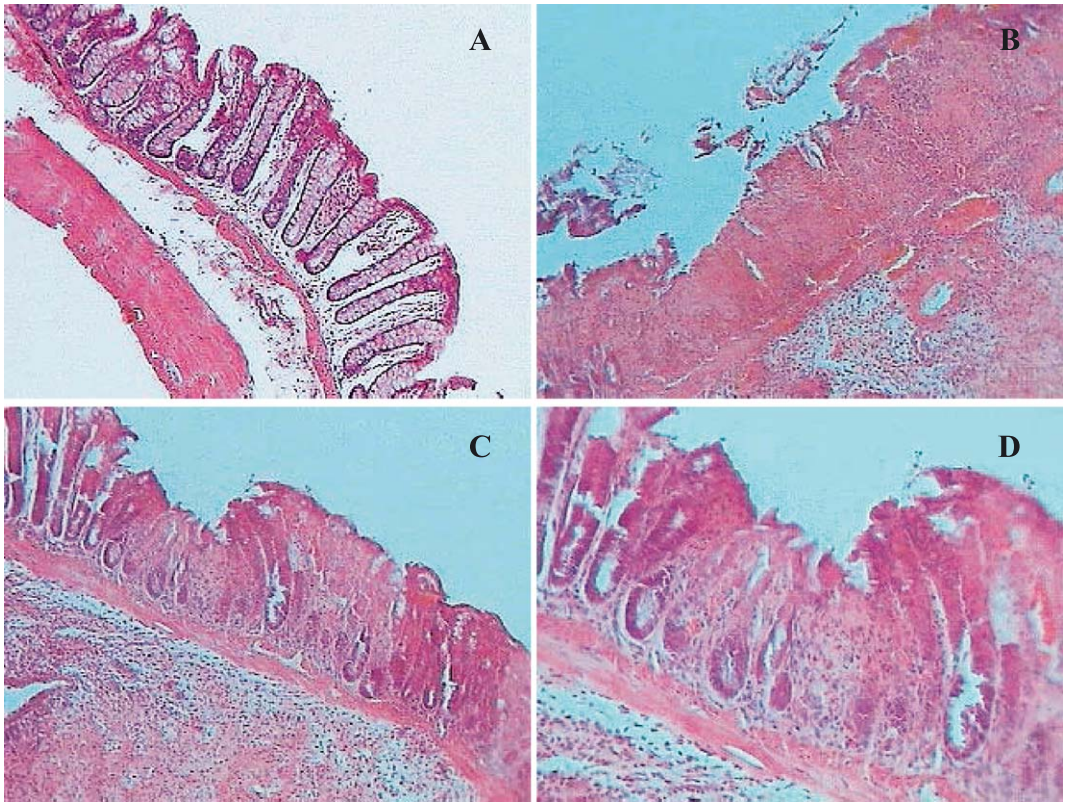

Fig. 2. Acute colitis model induced by TNBS: effect of rofecoxib on colon injury. Histological appearance of rat colonic mucosa: sham (A), and treated with TNBS 10 mg/animal (B), and rofecoxib 1.25 mg/kg (C). Histopathological features of the colon in association with colitis. (A) No histological modification was present in the sham animals. (B) Mucosal injury was produced after TNBS administration, characterized by necrosis of epithelium, focal ulceration of the mucosa and diffuse infiltration of inflammatory cells in the mucosa and submucosa. (C and D) Treatment with rofecoxib (1.25 mg/kg) reduced the morphological alterations associated with TNBS administration, protecting the mucosal architecture. H–E: Hematoxylin and eosin stain. Original magnification 10× (A, B and C) and 20× (D).

as the amount of enzyme present that produced a change in absorbance of 1.0 U/min at 37 °C in the final reaction volume containing sodium acetate. Results were expressed as U/mg tissue protein.

2.7. Production of prostaglandin E_2 and D_2

Colonic mucosa was excised and rapidly rinsed with ice-cold saline. The tissue was weighed and homogenized in triethylammonium phosphate (TEAP) buffer (pH 3.24) which contained a cyclo-oxygenase inhibitor, Inyesprin®. The homogenate was centrifuged (3000 rpm, 10 min, 4 °C) and the supernatant was removed. Prostaglandin D_2 was methoximated because of its chemical instability and rapid degradation. Later, the supernatant was passed through a reverse-phase octadecylsilica C18 Sep Pak cartridge, which was washed with 10 ml distilled water, 10 ml 15% ethanol, 10 ml hexane and 10 ml ethylacetate; the eluate was collected. Each fraction was evaporated with ethylacetate, and the dry residue was redissolved in ethanol. Prostaglandin E_2 and D_2 were measured with a competitive enzyme immunoassay kit (Assay Designs and Cayman Chemical, respectively). Results are expressed as pg/mg tissue.

2.8. Measurement of interleukin-1β production

The mucosal level of this cytokine was assayed by using a commercially available interleukin-1β enzyme-immunometric assay (EIA) kit (Titerzyme® EIA rat interleukin-1β, Assay Designs). Briefly, colonic mucosal samples kept at − 70 °C were weighed and homogenized, after thawing, in 10 volumes of assay buffer. They were centrifuged at 3800 rpm for 20 min, at 4 °C. Then 100 μl of the supernatant and 100 μl of standard and assay buffer were added to the wells of a microtiter plate with an immobilized polyclonal antibody to rat interleukin-1β. After incubation at 37 °C for 1 h, the excess sample or standard was washed out and a monoclonal antibody to rat interleukin-1β coupled to horseradish peroxidase was added. This labelled antibody binds to the rat interleukin-1β captured on the plate. After a short incubation, the excess labelled antibody was washed out and substrate was added. The substrate reacts with the labelled antibody bound to the rat interleukin-1β captured on the plate. The colour generated with the substrate was read at 450 nm in a microplate reader (Labysistem Multiskan EX), and is directly proportional to the concentration of rat interleukin-1β in either standard or sample. The interleukin-1β content is expressed as interleukin-1β/mg tissue.

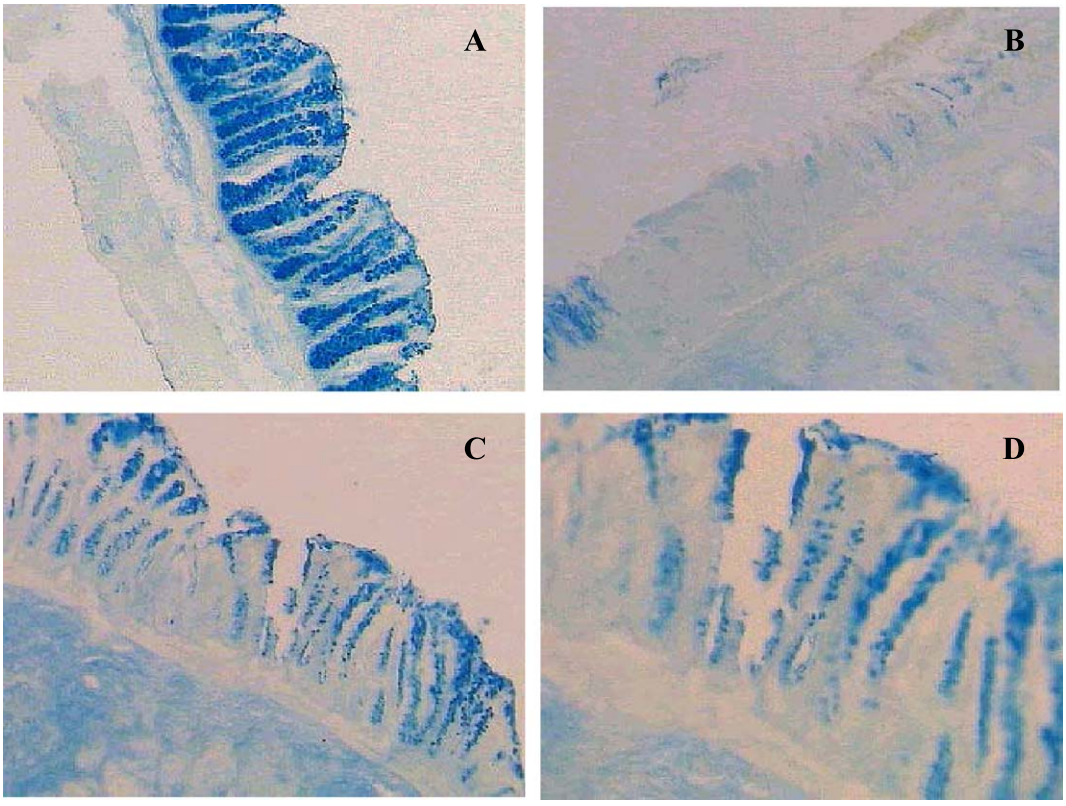

Fig. 3. Acute colitis model induced by TNBS: effect of rofecoxib on colon injury. Histological appearance of rat colonic mucosa after Alcian blue stain (AB): sham (A), and treated with TNBS 10 mg/animal (B), and rofecoxib 1.25 mg/kg (C and D, respectively). Some areas showed accumulation of mucus and cell remnants; however, Alcian blue positive–positive cells were less numerous, and the mucin layer of the epithelium was missing (B). Original magnification 10× (A, B and C) and 20× (D).

2.9. Apoptosis

Cytoplasmic DNA fragments, which are an indicator of apoptosis, were measured with a DNA cell death detection enzyme-linked immunosorbent assay (ELISA) PLUS KIT (Roche Diagnostics) according to the manufacturer's instructions.

2.10. Statistical analysis

All values in the figures and text are expressed as arithmetic means ± standard error (S.E.M.) of the mean. Data were evaluated with Graph Pad Prism® Version 2.01 software. The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA), followed by Fisher's test and the Mann–Whitney *U*-test. *P* values of <0.05 were considered statistically significant. In the experiment involving histology or immunohistochemistry, the figures shown are representative of at least six experiments performed on different days.

3. Results

Forty-eight hours after intracolonic administration of TNBS and ethanol, rats showed prostration, piloerection and hypomotility. Macroscopic inspection of the cecum, colon and rectum showed evidence of damage, namely, the presence of mucosal congestion, extensive disruption, and linear and deep haemorrhagic ulcerations. In this experimental group, body weight loss was greater than in the sham animals. The TNBS-treated animals became anorexic, with a pronounced decrease in average food intake compared to that of the vehicle-treated group (Table 1). Transmural necrosis and inflammatory masses in the descending colon were observed. Lesions in the distal colon were quantified with a macroscopic damage score (Fig. 1). A significant increase in the weight/length of the rat colon, an indicator of inflammation, was also observed in TNBS- and ethanol-treated rats (261.8 ± 34.5 mg/cm) in comparison with vehicle-treated rats (Table 1).

The histopathological features included transmural necrosis, oedema and diffuse inflammatory cell infiltration in the mucosa. There was focal ulceration of the colonic mucosa extending through the muscularis mucosae, desquamated areas and loss of the epithelium with mucin depletion. The architecture of the crypts was distorted and the lamina propria was thickened in peripheral areas of distorted crypts, especially in basal areas. An infiltrate consisting of polymorphonuclear leukocytes, lymphocytes, and eosinophils was observed. There was also granulation tissue in the submucosa (Fig. 2B). Some areas showed accumulation of mucus and cell remnants; however, Alcian blue positive-cells were less numerous. In addition, the mucin layer of the epithelium was missing (Fig. 3B).

Treatment of rats with rofecoxib significantly attenuated the extent and severity of the colonic injury, reducing the macroscopic damage score ($P<0.05$), although this effect was not dose dependent (Fig. 1). Histologically, there was attenuation of the extent and severity of the histological signs of cell damage and we did not see inflammatory cells in the lamina propria (Fig. 2C and D). In some areas, the epithelium remained intact and the mucin layer was clearly visible. Alcian blue-positive cells were less numerous; however, in ulcerative areas, exfoliation of epithelial cells, dilated crypts, inflammatory cells and congestion vascular were observed (Fig. 3C and D).

Table 2
Myeloperoxidase activity (MPO, U/mg tissue protein) and interleukin 1-beta levels (IL-1β, pg/mg tissue) after rofecoxib administration (1.25, 2.5 and 5 mg/kg) in rats with acute colitis produced by TNBS intracolonic instillation (10 mg/animal)

Group	*n*	MPO (U/mg tissue protein)	*n*	IL-1β (pg/mg tissue)
Sham	11	5.66 ± 0.63	7	0.08 ± 0.02
EtOH	15	7.12 ± 0.66	6	1.10 ± 0.32[a]
TNBS	14	8.89 ± 0.94[a]	5	3.28 ± 0.40[b,c]
R 1.25	9	5.45 ± 1.09[d]	9	0.25 ± 0.08[e]
R 2.5	8	6.94 ± 0.47	11	0.60 ± 0.11[e]
R 5	11	7.70 ± 0.84	11	0.58 ± 0.13[e]

Colonic mucosal myeloperoxidase activity and interleukin 1-beta levels were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham group, trinitobenzene sulphonic acid group and ethanol group), or in the presence of rofecoxib (1.25, 2.5 and 5 mg/kg/day). Data are expressed as means ± S.E.M. The myeloperoxidase activity and interleukin 1-β levels of colonic mucosa were quantified as described in Materials and methods and are expressed as U/mg tissue protein and pg/mg tissue, respectively.

[a] $P<0.05$ significantly different from sham.
[b] $P<0.001$ significantly different from sham.
[c] $P<0.001$ significantly different from EtOH.
[d] $P<0.05$ significantly different from TNBS.
[e] $P<0.001$ significantly different from TNBS.

Table 3
Prostaglandin E_2 (PGE_2, pg/mg tissue) and D_2 (PGD_2, pg/mg tissue) after rofecoxib administration (1.25, 2.5 and 5 mg/kg) in rats with acute colitis produced by TNBS intracolonic instillation (10 mg/animal)

Group	*n*	PGE_2 (pg/mg tissue)	*n*	PGD_2 (pg/mg tissue)
Sham	8	530.17 ± 65.70	7	2414.34 ± 249.06
EtOH	9	712.22 ± 129.41	9	2234.00 ± 227.83
TNBS	11	1165.73 ± 163.56[a]	10	1752.22 ± 231.45
R 1.25	10	704.36 ± 116.49[b]	11	1678.90 ± 182.28
R 2.5	10	850.75 ± 81.83[b]	12	1511.5 ± 200.23
R 5	9	719.11 ± 108.92[b]	10	1789.89 ± 174.75

Prostanoid synthesis in the colonic tissue was quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham group, trinitobenzene sulphonic acid group and ethanol group), or in the presence of rofecoxib (1.25, 2.5 and 5 mg/kg/day). Data are expressed as means ± S.E.M. The synthesis of prostanoids was quantified as described in Materials and methods and is expressed as pg/mg tissue.

[a] $P<0.01$ significantly different from sham.
[b] $P<0.05$ significantly different from TNBS.

As shown in Table 2, myeloperoxidase activity was significantly ($P<0.05$) increased in TNBS-treated animals compared with sham animals (8.98 ± 0.94 and 5.66 ± 0.63 U/mg tissue protein, respectively). These data are consistent with the histological findings. Treatment of TNBS-treated rats with rofecoxib (1.25 mg/kg) significantly reduced the degree of polymorphonuclear neutrophil infiltration. The levels of interleukin-1β were significantly elevated in the colon 48 h after TNBS instillation. In contrast, the levels of this cytokine were significantly lower ($P<0.001$) in rats treated with rofecoxib at all doses.

Our data showed that prostaglandin E_2 content increased significantly ($P<0.01$) in the colonic mucosa of the TNBS group compared with that of sham animals (Table 3). In addition, under our experimental conditions, all the tested doses of rofecoxib had no effect on the basal prostaglandin E_2 content of the colon but suppressed significantly ($P<0.05$ vs. TNBS) the increased prostaglandin E_2 levels to 704.36 ± 116.49 pg/mg tissue with the lowest dose (1.25 mg/kg), although this effect was not dose dependent. In contrast, treatment with rofecoxib did not have a statistically significant effect on prostaglandin D_2 concentration (Table 3).

In normal colon, specific immunoreactivity for cyclo-oxygenase-1 was seen in the surface epithelium. In the upper half of the crypts, mononuclear cells of the lamina propria mucosae and the regional lymphatic nodules as well as cells of the lamina muscularis mucosae showed cyclo-oxygenase-1 specific immunoreactivity (Fig. 4B). In the basal part of the crypts, cyclo-oxygenase-1 expression was restricted to individual cells, which according to morphological criteria were endocrine cells, a specialized epithelial cell type of the lower crypt (Fig. 4C).

Cyclo-oxygenase-2 specific immunolabeling was occasionally observed in colonocytes of the normal surface epithelium of matched control colon, as shown in Fig. 5B.

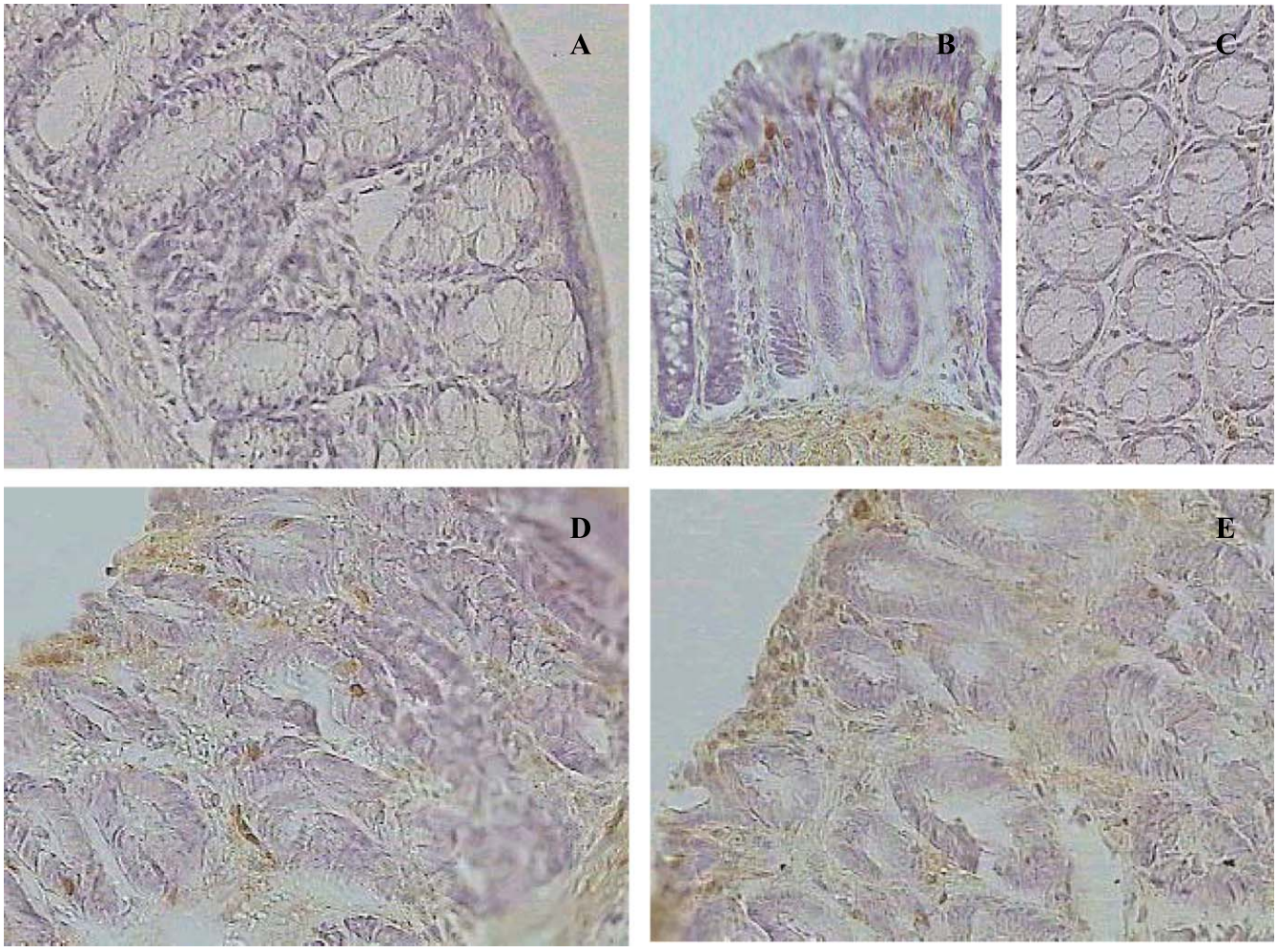

Fig. 4. Immunohistochemical localization of cyclooxygenase-1 isoenzyme in sections of colon. To assess the specificity of the immunoreaction, control sections were incubated without the primary antibody: negative control (A). Cyclooxygenase-1 expression in endocrine cells is particularly evident in this image of normal colonic mucosa (B). In normal colon, colonocytes of the upper half of the crypts were found to be cyclooxygenase-1-positive (C). Cyclooxygenase-1 expression in the colon of TNBS-control rats (D). Cyclooxygenase-1 expression of inflamed colon treated with rofecoxib 1.25 mg/kg (E). Original magnification 40×.

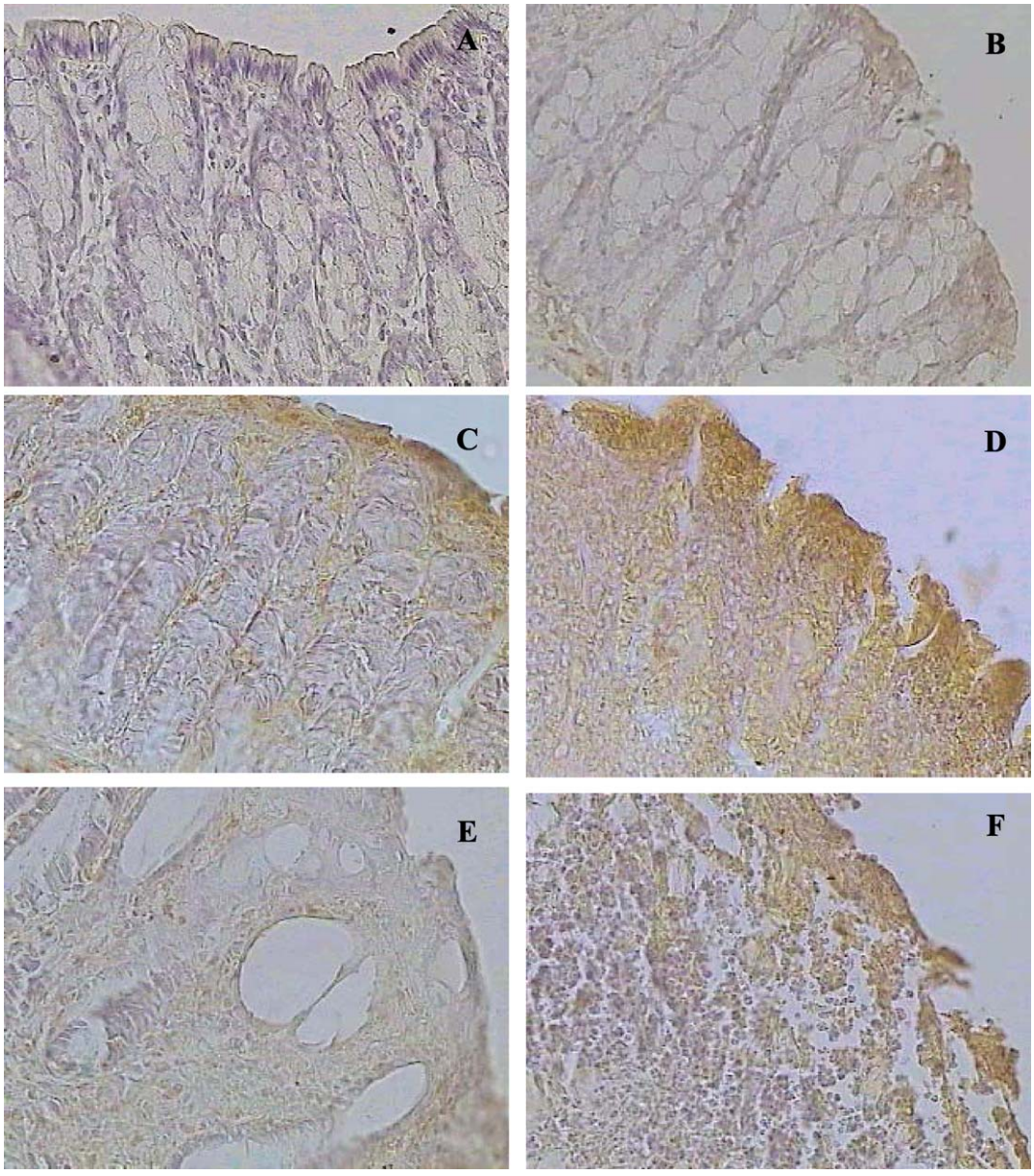

Fig. 5. Immunohistochemical localization of cyclooxygenase-2 isoenzyme in sections of colon. Control section was incubated without the primary antibody: negative control (A). Cyclooxygenase-2 expression in normal colonic mucosa (B). Cyclooxygenase-2 is strongly expressed in the colon of TNBS-control rats (C and D). Cyclooxygenase-2 expression was decreased in apical epithelial cells of inflamed colon treated with rofecoxib 1.25 mg/kg (E and F). Original magnification 40×.

Compared with normal colon, significant changes in the cellular distribution of cyclo-oxygenase-1 and cyclo-oxygenase-2 were observed in animals treated with TNBS, in that colonocytes of the surface and the crypt epithelium were only weakly labelled by the cyclo-oxygenase-1 specific antiserum, whereas prominent cyclo-oxygenase-1 expression was found in cells of the inflammatory infiltrate (Fig. 4D). Intense cyclo-oxygenase-2 immunostaining was observed in cells of the surface epithelium, while crypt epithelial cells were negative for cyclo-oxygenase-2, which was also prominent in mononuclear cells of the inflammatory infiltrate (Fig. 5C and D). Compared with inflamed colon, in rofecoxib-treated colon there was no significant change in the cellular localization of cyclo-oxygenase-1 (Fig. 4E). However, cyclo-oxygenase-2 expression was decreased in apical epithelial cells of inflamed colon treated with rofecoxib (Fig. 5E and F).

Since cyclo-oxygenase-2 inhibitors have been found to modulate apoptosis, we wished to know what their effects were in colonic mucosa under early acute inflammatory conditions. To this end, we used an ELISA that specifically detected cytoplasmic histone-associated DNA fragments, mononucleosomes, and oligonucleosomes. As shown in Fig. 6, apoptosis was rarely observed in the colonic mucosa of sham animals. DNA fragmentation was significantly ($P<0.05$) increased in TNBS-treated rats, but in

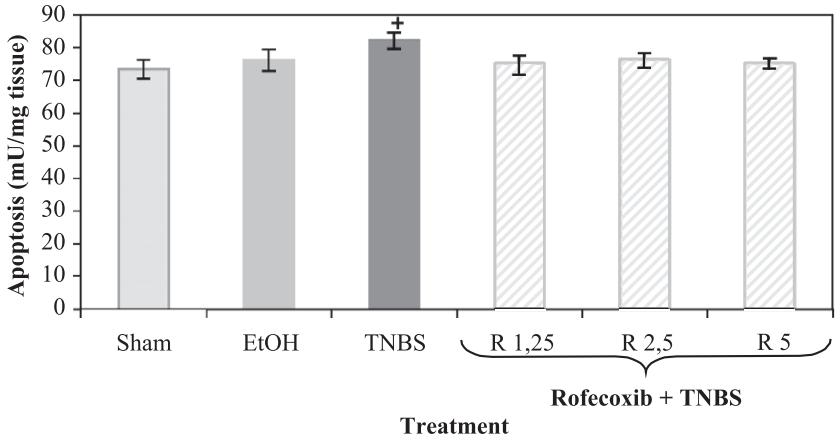

Fig. 6. Apoptosis observed in the colonic mucosa after acute colitis induced by TNBS (10 mg/animal). Apoptosis were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham group, trinitobenzene sulphonic acid group and ethanol group), or in the presence of rofecoxib (1.25, 2.5 and 5 mg/kg/day). Data are expressed as means ± S.E.M. There were no significant differences between any of the groups ($^{+}P < 0.05$ vs. sham group).

contrast there were no significant changes in rofecoxib-treated rats.

4. Discussion

The present study confirmed that treatment with the cyclo-oxygenase-2 inhibitor, rofecoxib, reduced the inflammation and the acute colonic damage induced by TNBS and ethanol, as verified by macroscopic, histological and biochemical data.

The role of prostanoids in the intestinal inflammatory process is not completely understood. During the course of inflammatory bowel disease and experimental colitis, some prostanoids are formed and negatively modulate extension of the disease (Carty et al., 2000). Our results showed that the elevated production of prostaglandin E_2 and up-regulation of cyclo-oxygenase-2 were clearly induced by TNBS. Administration of the cyclo-oxygenase-2 inhibitor, rofecoxib, produced a significant reduction in tissue prostanoid production and very weak cyclo-oxygenase-2-immunoreactivity. It is well known that prostaglandin E_2 is an important inflammatory mediator that induces epithelial cell chloride secretion, which results in the diarrhoea observed in inflammatory bowel disease; the diarrhoea may lead to weight loss during inflammatory bowel disease. Endogenous prostaglandin E_2 is produced by mononuclear cells in the lamina propria and is dependent on cyclo-oxygenase-2 expression. It is thought to be the most potent mediator of inflammation because of vasodilator effects during the inflammatory process, but it also modulates the intestinal immune response, including differentiation of T cells and the production and release of proinflammatory cytokines such as TNF-á and interleukin (IL)-1β (Newberry et al., 1999). Our data, which are in accord with previous studies (Guo et al., 2001; Kankuri et al., 2001), suggest that the increased prostaglandin E_2 production during TNBS-induced acute colitis is dependent upon the activity of cyclo-oxygenase-2. Thus, the protective role of cyclo-oxygenase-2 inhibitors is largely due to the inhibition of the strongly induced cyclo-oxygenase-2.

There is good evidence that an enhanced formation of reactive oxygen species contributes to the pathophysiology of inflammatory bowel disease (Guo et al., 1999; Kruidenier and Verspaget, 2002). Quantitatively, the principal free radical in tissues is superoxide anion (O_2^-), which is converted to the secondary oxidant H_2O_2 by superoxide dismutase. O_2^- can be produced by activated neutrophils through NADPH oxidase, which reduces molecular oxygen to the O_2^- radical, and through the enzyme myeloperoxidase. In our experiments, the increase in myeloperoxidase activity in the colon was reduced by rofecoxib. This beneficial effect is in agreement with the results of previous reports (Cuzzocrea et al., 2001; Kankuri et al., 2001) showing that other cyclo-oxygenase-2 inhibitors, such as celecoxib or nimesulide, markedly reduce cellular infiltration and inflammation of the colon, probably by a mechanism related to inhibition of prostaglandin E_2 production by the cyclo-oxygenase-2 pathway.

In this study, the pathogenesis of colitis was also associated with increased interleukin-1β levels. Interleukin-1β is a key immunoregulatory cytokine that amplifies the inflammatory response by activating a cascade of immune cells (Sartor, 1994). Interleukin-1β in high doses produces epithelial cell necrosis, oedema, neutrophil infiltration and, globet cell depletion. Blocking of the action of endogenous interleukin-1 attenuates acute and chronic experimental colitis and its systemic complications (Rogler and Andus, 1998; Siegmund, 2002). Our results showed a significant suppression of interleukin-1β in the rofecoxib groups and are consistent with those obtained with other cyclo-oxygenase-2 inhibitors (Karmeli et al., 2000; Cuzzocrea et al., 2001). We have demonstrated that (1) increased prostaglandin E_2 production is associated with macroscopic damage, (2) cyclo-oxygenase-2 protein expression is increased in

colon following the induction of colitis and (3) the high expression of cyclo-oxygenase-2 in the colon is associated with leukocyte infiltration (measured as myeloperoxidase activity) and cytokine production. Therefore, the ability of rofecoxib to partially reduce the inflammatory cell infiltrate in the colon could in part explain the observed reduction in the levels of cyclo-oxygenase-2 and cytokine production.

Prostaglandin D_2 is the major prostaglandin produced by mucosal mast cells and has been suggested to exert anti-inflammatory effects, playing an important role in down-regulating colon inflammation in rats. In a previous study by Ajuebor et al. (2000), prostaglandin D_2 synthesis was elevated in relation to controls within 1–3 h of the induction of colitis. Nevertheless, our results showed no significant changes in this prostanoid in TNBS- and rofecoxib-treated animals, suggesting that 48 h after intracolonic administration of TNBS, prostaglandin D_2 seems not to be involved in the acute stage of lesion and inflammation formation induced by TNBS.

Our histological results also revealed that rofecoxib increased the amount of mucus stained by Alcian blue (acid glucoproteins such as sialomucins) in colon mucosa. The protective effect of mucus as an active barrier may be attributed largely to its viscous and gel-forming properties, which are due to the glycoprotein constituents of mucin. Alcian blue-positive cells seem to be associated with regenerative processes of the mucosa (Alarcón de la Lastra et al., 1994; Villegas et al., 2003), while reduction in the amount stained has been related to decreased resistance of the mucosa and paralleled by alterations in the normal pattern of maturation of mucin in globet cells (Torres et al., 1999). TNBS-induced colitis results in acute necrosis, which is likely caused by oxidative damage (Morris et al., 1989). It has been reported that immobilization stress in rats causes significant increases in colonic prostaglandin E_2, and mucin release and faecal pellet output and causes an approximately 10-fold increase in colonic mucosal levels of cyclo-oxygenase-2 mRNA (Castagliuolo et al., 1996). In addition, it has been demonstrated that elevated levels of prostaglandins may contribute to the recognized decrease in intracellular mucin stores and to changes in the type of mucin species present at sites of mucosal inflammation in patients with ulcerative colitis (Phillips et al., 1993). Accordingly, we suggest that the high levels of colonic prostaglandin E_2 produced by cyclo-oxygenase-2 in TNBS-treated rats may contribute to mucus depletion, and thus its inhibition by rofecoxib may explain the increase in the amount of mucus.

It has been reported that increased apoptosis is the main cause of epithelial loss in the crypts of patients with active ulcerative colitis (Iwamoto et al., 1996). Apoptosis enhancement may have a beneficial consequence by eliminating heavily damaged mutation-prone cells in which DNA repair is at risk of being suppressed (Tardieu et al., 2000). Previous studies have shown significant apoptosis in colonic epithelial cells during mild acute inflammation induced by dextran sulphate sodium (Tardieu et al., 2000) and in TNBS-induced colitis (Yue et al., 2001). These findings are in accord with the present study, in which colonic cell death was associated with apoptosis in the colon lesion 48 h after intracolonic administration of TNBS. An increase in apoptosis linked to cyclo-oxygenase-2 inhibition has already been documented in colonic cells in vitro (Hara et al., 1997; Arico et al., 2002) and in vivo inflammatory conditions (Tardieu et al., 2000). For example, some selective cyclo-oxygenase-2 inhibitors, i.e. celecoxib, nimesulide, NS-398, are effective in inducing the characteristic features of apoptosis, including DNA fragmentation, terminal deoxynucleotidyl transferase-mediated dUTP (TUNEL)-positivity and caspase-3/7 activation, in colon carcinoma cells (Yamazaki et al., 2002) and rheumatoid synovial fibroblasts (Kusunoki et al., 2002); however rofecoxib has no effect (Yamazaki et al., 2002), suggesting that the proapoptotic effect of cyclo-oxygenase-2 inhibitors is unrelated to inhibition of cyclo-oxygenase-2. It is possible that other mechanisms are implicated, such as the activation of peroxisome proliferator-activated receptor or an anti-angiogenetic action. In our study, we clearly showed that rofecoxib did not influence DNA fragmentation of colonic cells in TNBS-treated rats. However, in an in vitro study, rofecoxib resulted in a dose-dependent increase in apoptosis and dose- and time-dependent inhibition of cell proliferation of 3LL tumour cells. (Qadri et al., 2002). This discrepancy may be explained by differences in the in vivo and in vitro experimental conditions.

Taken together, our data confirm that the strong expression of cyclo-oxygenase-2 in the initial stage of experimental ulcerative colitis seems to act as a promoter of the colonic lesion and inflammation development, and that treatment with the cyclo-oxygenase-2 inhibitor, rofecoxib, exerts protective effects against inflammatory oedema formation in response to acute experimentally induced colitis. Rofecoxib reduced excessive prostaglandin E_2 production and overexpression of cyclo-oxygenase-2. These anti-inflammatory effects seem to be related to an impairment of neutrophil function, the absence of up-regulation of interleukin-1β, and an increase in mucus production in colon mucosa. These data suggest that cyclo-oxygenase-2-selective inhibitors may have a therapeutic role in ulcerative colitis, and thus most patients with inflammatory bowel disease could take selective cyclo-oxygenase-2 inhibitors without their disease flaring up.

References

Agoff, S.N., Brentnall, T.A., Crispin, D.A., Taylor, S.L., Raaka, S., Haggitt, R.C., Reed, M.W., Afonina, I.A., Rabinovitch, P.S., Stevens, A.C., Feng, Z., Bronner, M.P., 2000. The role of cyclo-oxygenase 2 in ulcerative colitis-associated neoplasia. Am. J. Pathol. 157, 737–745.

Ajuebor, M.N., Singh, A., Wallace, J.L., 2000. Cyclooxygenase-2-derived prostaglandin D2 is an early anti-inflammatory signal in experimental colitis. Am. J. Physiol. 279, G238–G244.

Alarcón de la Lastra, C., Martín, M.J., Motilva, V., 1994. Antiulcer and gastroprotective effect of quercetin: a gross and histologic study. Pharmacology 48, 56–62.

Arico, S., Pattingre, S., Bauvy, C., Gane, P., Barbat, A., Codogno, P., Ogier-Denis, E., 2002. Celecoxib induces apoptosis by inhibiting 3-phosphoinositide-dependent protein kinase-1 activity in the human colon cancer HT-29 cell line. J. Biol. Chem. 277, 27613–27621.

Bobin-Dubigeon, C., Collin, X., Grimaud, N., Robert, J.M., Le Baut, G., Petit, J.Y., 2001. Effects of tumor necrosis factor-α synthesis inhibitors on rat trinitrobenzene sulphonic acid-induced chronic colitis. Eur. J. Pharmacol. 431, 103–110.

Bonner, G.F., 2002. Using COX-2 inhibitors in IBD: anti-inflammatories inflame a controversy. Am. J. Gastroenterol. 97, 783–785.

Bonner, G.F., Walczak, M., Kitchen, L., Bayona, M., 2000. Tolerance of nonsteroidal anti-inflammatory drugs in patients with inflammatory bowel disease. Am. J. Gastroenterol. 95, 1055–1058.

Campbell, N.B., Jones, S.L., Blikslager, A.T., 2002. The effects of cyclooxygenase inhibitors on bile-injured and normal equine colon. Equine Vet. J. 34, 493–498.

Carty, E., De Brabander, M., Feakins, R.M., Rampton, D.S., 2000. Measurement of in vivo rectal mucosal cytokine and eicosanoid production in ulcerative colitis using filter paper. Gut 46, 487–492.

Castagliuolo, I., Lamont, J.T., Qiu, B., Fleming, S.M., Bhaskar, K.R., Nikulasson, S.T., Kornetsky, C., Pothoulakis, C., 1996. Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells. Am. J. Physiol. 271, G884–G892.

Cuzzocrea, S., Mazzon, E., Serraino, I., Dugo, L., Centorrino, T., Ciccolo, L., Sautebin, L., Caputi, A.P., 2001. Celecoxib, a selective cyclo-oxygenase-2 inhibitor reduces the severity of experimental colitis induced by dinitrobenzene sulphonic acid in rats. Eur. J. Pharmacol. 431, 91–102.

Dannhart, G., Kiefer, W., 2001. Cyclooxygenase inhibitors—current status and future prospects. Eur. J. Med. Chem. 36, 109–126.

Fiocchi, C., 1998. Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 115, 182–205.

Grisham, M.B., Beniot, J.N., Granger, D.N., 1990. Assessment of leucocyte in involvement during ischemia and reperfusion on the intestine. In: Packer, L., Glazer, A.E. (Eds.), Methods in Enzymology. Oxygen Radicals in Biological Systems. Academic Press, San Diego, pp. 729–741.

Guo, X., Wang, W.P., Ko, J.K., Cho, C.H., 1999. Involvement of neutrophils and free radicals in the potentiating effects of passive cigarette smoking on inflammatory bowel disease in rats. Gastroenterology 117, 884–892.

Guo, X., Liu, E.S., Ko, J.K., Wong, B.C., Ye, Y., Lam, S., Cho, C., 2001. Protective role of cyclooxygenase inhibitors in the adverse action of passive cigarette smoking on the initiation of experimental colitis in rats. Eur. J. Pharmacol. 411, 193–203.

Gupta, R.A., Dubois, N., 2001. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat. Rev. 1, 11–21.

Hara, A., Yoshimi, N., Niwa, M., Ino, N., Mori, H., 1997. Apoptosis induced by NS-398, a selective cyclooxygenase-2 inhibitor, in human colorectal cancer lines. Jpn. J. Cancer Res. 88, 600–604.

Iwamoto, M., Koji, T., Makiyama, K., Kobayashi, N., Nakane, P.K., 1996. Apoptosis of crypt epithelial cells in ulcerative colitis. J. Pathol. 180 (2), 152–159.

Kankuri, E., Vaali, K., Korpela, R., Paakkari, I., Vapaatalo, H., Moilanen, E., 2001. Effects of a COX-2 preferential agent nimesulide on TNBS-induced acute inflammation in the gut. Inflammation 25, 301–309.

Karmeli, F., Cohen, P., Rachmilewitz, D., 2000. Cyclo-oxygenase-2 inhibitors ameliorate the severity of experimental colitis in rats. Eur. J. Gastroenterol. Hepatol. 12, 223–231.

Khan, I., Al-Awadi, F.M., Thomas, N., Haridas, S., Anim, J.M., 2002. Cyclo-oxygenase-2 inhibition and experimental colitis: beneficial effects of phosphorothioated antisense oligonucleotide and meloxicam. Scand. J. Gastroenterol. 37, 1428–1436.

Kruidenier, L., Verspaget, H.W., 2002. Oxidative stress as a pathogenic factor in inflammatory bowel disease—radicals or ridiculous? Aliment. Pharmacol. Ther. 16, 1997–2015.

Kusunoki, N., Yamazaki, R., Kawai, S., 2002. Induction of apoptosis in rheumatoid synovial fibroblasts by celecoxib, but not by other selective cyclooxygenase 2 inhibitors. Arthritis Rheum. 46, 3159–3167.

Lesch, C.A., Kraus, E.R., Sanchez, B., Gilbertsen, R., Guglietta, A., 1999. Lack of beneficial effect of COX-2 inhibitors in an experimental model of colitis. Methods Find Exp. Clin. Pharmacol. 21, 99–104.

Mahadevan, U., Loftus, E.V., Tremaine, W.J., Sandborn, W.J., 2002. Safety of cyclooxygenase-2 inhibitors in inflammatory bowel disease. Am. J. Gastroenterol. 97, 910–914.

Morris, G.P., Beck, P.L., Herridge, M.S., Depew, W.T., Szewczuk, M.R., Wallace, J.L., 1989. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology 96 (3), 795–803.

Newberry, R.D., Stenson, W.F., Lorenz, R.G., 1999. Cyclooxygenase-2-dependent arachidonic acid metabolites are essential modulators of the intestinal immune response to dietary antigen. Nat. Med. 5, 900–906.

Nitta, M., Hirata, I., Toshina, K., Murano, M., Maemura, K., Hamamoto, N., Sasaki, S., Yamauchi, H., Katsu, K., 2002. Expression of the EP4 prostaglandin E2 receptor subtype with rat dextran sodium sulphate colitis: colitis suppression by a selective agonist, ONO-AE1-329. Scand. J. Immunol. 56, 66–75.

Phillips, T.E., Stanley, C.M., Wilson, J., 1993. The effect of 16,16-dimethyl prostaglandin E2 on proliferation of an intestinal goblet cell line and its synthesis and secretion of mucin glycoproteins. Prostaglandins Leukot. Essent. Fat. Acids 48, 423–428.

Qadri, S.S., Wang, J.H., Redmond, K.C., O' Donnell, A.F., Aherne, T., Redmond, H.P., 2002. The role of COX-2 inhibitors in lung cancer. Ann. Thorac. Surg. 74, 1648–1652.

Reuter, B.K., Asfaha, S., Buret, A., Sharkey, K.A., Wallace, J.L., 1996. Exacerbation of inflammation-associated colonic injury in rat through inhibition of cyclooxygenase-2. J. Clin. Invest. 98, 2076–2085.

Rogler, G., Andus, T., 1998. Cytokines in inflammatory bowel disease. World J. Surg. 22 (4), 382–389.

Sartor, R.B., 1994. Cytokines in intestinal inflammation: pathophysiological and clinical considerations. Gastroenterology 106, 533–539.

Siegmund, B., 2002. Interleukin-1beta converting enzyme (caspase-1) in intestinal inflammation. Biochem. Pharmacol. 64, 1–8.

Simon, L.S., 1998. Biology and toxic effects of nonsteroidal anti-inflammatory drugs. Curr. Opin. Rheumatol. 10, 153–158.

Singer, I.I., Kawka, D.W., Schloemann, S., et al., 1998. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. Gastroenterology 115, 297–306.

Tardieu, D., Jaeg, J.P., Deloly, A., Corpet, D.E., Cadet, J., Petit, C.R., 2000. The COX-2 inhibitor nimesulide suppresses superoxide and 8-hydroxydeoxyguanosine formation, and stimulates apoptosis in mucosa during early colonic inflammation in rats. Carcinogenesis 21, 973–976.

Torres, M.I., García-Martín, M., Fernández, M.I., Nieto, N., Gil, A., Ríos, A., 1999. Experimental colitis induced by trinitrobenzenesulphonic acid. An ultrastructural and histochemical study. Dig. Dis. Sci. 44, 2523–2529.

Vane, J.R., Botting, R.M., 1998. Mechanism of action of nonsteroidal anti-inflammatory drugs. Am. J. Med. 104, 2S–8S.

Villegas, I., La Casa, C., Orjales, A., Alarcón de la Lastra, C., 2003. Effects of dosmalfate, a new cytoprotective agent, on acute and chronic trinitrobenzene sulphonic acid-induced colitis in rats. Eur. J. Pharmacol. 460, 209–218.

Warner, T.D., Giuliano, F., Vojnovic, I., Bukada, A., Mitchell, J.A., Vane, J.R., 1999. Non-steroidal drug selectivities for cyclooxygenase-1 rather than cyclooxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis. Proc. Natl. Acad. Sci. U. S. A. 96 (13), 7563–7568 (Find. Exp. Clin. Pharmacol. 21, 99-104.).

Yamazaki, R., Kusunoki, N., Matsuzaki, T., Hashimoto, S., Kawai, S., 2002. Selective cyclooxygenase-2 inhibitors show a differential ability to inhibit proliferation and induce apoptosis of colon adenocarcinoma cells. FEBS Lett. 531, 278–284.

Yue, G., Lai, P.S., Yin, K., Sun, F.F., Nagele, R.G., Liu, X., Linask, K.K., Wang, C., Lin, K.T., Wong, P.Y., 2001. Colon epithelial cell death in 2,4,6-trinitrobenzenesulfonic acid-induced colitis is associated with increased inducible nitric-oxide synthase expression and peroxynitrite production. J. Pharmacol. Exp. Ther. 297 (3), 915–925.