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Rosiglitazone, a PPARγ ligand, modulates signal transduction pathways during the development of acute TNBS-induced colitis in rats

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Abstract

Recent studies have shown that peroxisome proliferator-activated receptor gamma (PPARγ), a highly nuclear receptor expressed in the colon, may participate in the control of inflammation, especially in regulating the production of immunomodulatory and inflammatory mediators, cellular proliferation and apoptosis. In order to delve into the anti-inflammatory mechanisms and signalling pathways of PPARγ agonists, we have studied the effects of rosiglitazone, a PPARγ agonist on the extent and severity of acute ulcerative colitis caused by intracolonic administration of 2,4,6-trinitribenzene sulfonic acid (TNBS) in rats. The inflammatory response was assessed by gross appearance, myeloperoxidase (MPO) activity, tumour necrosis factor α (TNF-α) levels and a histological study of the lesions. We determined prostaglandin E2 production as well as the cyclooxygenases (COX)-1 and -2 expressions by immunohistochemistry and Western blotting. The nuclear factor kappa (NF-κB) p65 and p38 mitogen-activated protein kinase (MAPK) expression levels were also measured by Western blotting. Finally, since PPARγ agonists modulate apoptosis, we tried to clarify its effects under early acute inflammatory conditions. Inflammation following TNBS induction was characterized by increased colonic wall thickness, edema, diffuse inflammatory cells infiltration, necrosis reaching an ulcer index (UI) of 9.66±0.66 cm^2 and increased MPO activity and TNF-α colonic levels. Rosiglitazone treatment significantly reduced the morphological alteration associated with TNBS administration and the UI with the highest dose. In addition, the degree of neutrophil infiltration and the cytokine levels were significantly ameliorated. Rosiglitazone significantly reduced the rise in the prostaglandin (PG) E_2 generation compared with TNBS group. The COX-1 levels remained stable throughout the treatment in all groups. The COX-2 expression was elevated in TNBS group; however rosiglitazone administration reduced the COX-2 overexpression. A high expression of NF-κB p65 and p38 MAPK proteins appeared in colon mucosa from control TNBS-treated rats; nevertheless, PPARγ agonist treatment drastically decreased them. There were no significant changes in apoptosis after rosiglitazone treatment when compared to TNBS group. In conclusion, rosiglitazone seems to modulate the acute colitis through NF-κB p65 and p38 MAPK signalling pathways.

Keywords: Apoptosis; MAPK (mitogen-activated protein kinase); Neutrophil; NF-κB (nuclear factor kappa B); PPARγ (peroxisome proliferator-activated receptor gamma); Prostaglandin(PG)E_2; TNBS (trinitrobenzenesulphonic acid); (Rat)

1. Introduction

The peroxisome proliferator-activated receptors (PPAR) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors that are related to retinoid, steroid and thyroid hormone receptors. PPARs regulate gene expression by binding to retinoid X receptors (RXR) as heterodimers. To date, three isoforms, encoded by separated genes, have been identified: PPARα, PPARβ or PPARδ and PPARγ. PPARγ has classically been characterized for its implications in adipocyte differentiation and fat metabolism. Recent studies have shown that thiazolidindiones (TZD) may participate in inflammation control, especially in regulating the production of immunomodulatory and inflammatory mediators (Cabrero et al., 2002; Na and Surh, 2003; Pershadsingh, 2004; Alarcón de la Lastra et al., 2004). Moreover, PPARγ induces a decrease in pro-inflammatory cytokines expression by antagonizing the activities of c-jun-NH_2-terminal kinase (JNK) and

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p38 MAPK *in vivo* (Desreumaux et al., 2001) and interfering with the transcription factor activation such as nuclear factor NF-κB, signal transducers and transcription activators (STAT), activating protein 1 (AP-1), and the nuclear factor of activated T-cells (NFAT), all of which regulate cytokine gene expression (Dubuquoy et al., 2002).

PPARγ is highly expressed in the colon, mainly in the crypts and surface of epithelial cells (Lytle et al., 2005), as well as in macrophages and T and B lymphocytes (Desreumaux et al., 2001; Lytle et al., 2005); however, its levels are decreased during chronic inflammation in humans and animals (Lefebvre et al., 1999; Katayama et al., 2003).

Ulcerative colitis is a non-specific inflammatory disorder involving primarily the mucosa and submucosa of the colon. Activated immune cells, mainly represented by neutrophils, macrophages, and cytotoxic T cells, play the role of aggressors that attack and destroy the intestinal barrier directly through physical contact or indirectly through the release of reactive oxygen and nitrogen metabolites. Reactive oxygen species are now increasingly recognized to be involved in cell growth, signalling and gene expression (Szanto et al., 2005). Furthermore, reactive oxygen species can activate diverse downstream signalling pathways, such as mitogen-activated protein kinases (MAPKs) or the transcription factor nuclear factor kappa B (NF-κB), thus modulating a number of different steps in the inflammatory cascade. These include production of pro-inflammatory cytokines (tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, interferon (INF)-γ IL-12, and IL-6) in different cell-types, degranulation of neutrophils, as well as the expression of important determining parameters of colonic damage i.e. cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS) (Dubuquoy et al., 2002; Collino et al., 2006).

There are recent reports which document that PPARγ ligands decrease the degree of inflammation associated with experimental colitis. For example, we have previously reported that the PPARγ ligand, rosiglitazone, was able to suppress the degree of inflammation associated with chronic experimental colitis (Sanchez-Hidalgo et al., 2005). Cuzzocrea et al. (2003) demonstrated the protective effects of the endogenous PPARγ agonist, 15d-PGJ_2, on colon damage caused by dinitrobenzene sulfonic acid in the rat. Interestingly, Desreumaux et al. described the effectiveness of PPARγ ligands for the amelioration of 2,4,6,-trinitrobenzene sulfonic acid (TNBS)-induced acute colitis in $PPAR\gamma^{+/-}$ mice. Similarly, Su et al. (1999) and Takagi et al. (2002) reported the attenuation of colonic inflammation by troglitazone in mice. Basing ourselves upon these data there is no doubt that PPARγ may play a central role in anti-inflammatory responses in the colon. Nevertheless, there are many interesting questions regarding the molecular mechanisms for action and signalling pathways in ulcerative colitis. On the other hand, the novelty of this study is that the effects of the TZD, rosiglitazone, in colonic mucosa under acute TNBS-induced colitis in rats were tested for the first time as well as the involvement of the signalling pathways implicated in experimental acute inflammatory disease development. The inflammatory response was assessed by histology and myeloperoxidase (MPO) activity, as an index of quantitative inflammation and neutrophil infiltration in the mucosa. TNF-α production and histological and histochemical analysis lesions were also carried out. Prostaglandin (PG)E_2 generation and COX-1, COX-2, nuclear transcription factor NF-κB p65 and MAPK p38 expression were analyzed in order to gain a better insight into the action mechanism(s) of the observed protective effects of rosiglitazone. Finally, since PPARγ agonists have been found to modulate apoptosis in colitis-related colon carcinogenesis (Sanchez-Hidalgo et al., 2005; Kohno et al., 2001) our aim was to study their effects on acute TNBS-induced colitis in rats.

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 a controlled room (temperature 24–25 °C, humidity 70–75%, lighting regimen of 12L/12D) and were fed 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. 14 animals were randomly assigned to each group. 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. Briefly, rats were slightly anaesthetised with ether following a 24 h fast, and then a medical-grade polyurethane canal for enteral feeding (external diameter 2 mm) was inserted into the anus and the tip was advanced to 8 cm proximal to the anus verge. TNBS (Sigma-Aldrich Company Ltd., Spain) dissolved in 50% ethanol were 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 sham group received an enema of physiological saline instead of the TNBS solution, and ethanol group received 0.25 ml of 50% ethanol. Rosiglitazone (4–8 mg/kg p.o.; Sigma-Aldrich Company Ltd., Spain) was suspended in 0.9% saline solution and administered by gavage 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 animal). The animals were sacrificed, using an overdose of anaesthetic, 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, slightly cleaned in physiological saline to remove fecal residues and weighed. Macroscopic inflammation scores were assigned based on clinical features of the colon (Sanchez-Hidalgo et al., 2005). The presence of adhesions (score 0–2), and/or stool consistency (score 0–1) were evaluated according to the criteria of Bobin-Dubigeon et al. (2001). Pieces of inflamed colon were collected and frozen in liquid nitrogen to measure biochemical parameters.

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, sections of tissue were cut at 5 μm 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, Giemsa, and Alcian blue for histological evaluation of colonic damage, cell infiltration and mucus content respectively, according to standard protocols, and the slides were coded to prevent observer bias during evaluation. All tissue sections were examined in an Olympus BH-2 microscope for characterization of histopathological changes.

Photographs taken from colon samples were digitised using Kodak D290 Zoom camera Eastman Kodak Co., USA and Motic® Images 2000 release 1.1 (MicroOptic Industrial Group CO., LTD; B1 Series System Microscopes). Analysis of the figures was carried out by Adobe® Photoshop® Version 5.0 (Adobe Systems) image analysis program.

2.5. Immunohistochemical study

Immunohistochemical analysis was performed using the technique described in our previous article (Sanchez-Hidalgo et al., 2005). 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. All of them were treated with a buffered blocking solution (3% bovine serum albumin) for 15 min. Then, sections were co-incubated with primary antibodies for COX-1 and COX-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 phosphate-buffered saline (PBS) and co-incubated with secondary antibody (anti-sheep IgG, peroxidasic conjugated, Sigma, Spain) (1:500 in 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 a 3, 3′-diaminobenzidine solution in darkness at room temperature for 10 min. Sections were washed with Tris–HCl, stained with haematoxylin according to standard protocols and observed under an Olympus BH-2 microscope.

2.6. Assessment of leukocyte involvement

MPO activity was assessed as a marker of neutrophil 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 vol 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 vol 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. The mixture was incubated at 37 °C for 5 min and the reaction started by the addition of 0.3 mM H_2O_2.

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 MPO activity was defined 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 the acetate. Results were quantified as U/mg tissue.

2.7. TNF-α levels

Distal colon samples were weighed and homogenized, after thawing, in 0.3 ml phosphate buffer saline solution (PBS pH 7.2) at 4 °C. They were centrifuged at 15,300 ×*g* for 10 min. Mucosal TNF-α level was assayed with a quantitative TNF-α enzyme immunoassay kit (Quantikine® M, R&D Systems). The TNF-α values were expressed as pg/mg tissue.

2.8. Production of PGE_2

Colonic mucosa was excised and rapidly rinsed with ice-cold saline. The tissue was weighed and homogenized in 6 ml triethylammonium phosphate (TEAP) buffer (pH 3.24), which contained a COX inhibitor, lysine acetyl salicylate (Sigma-Aldrich Company Ltd., Spain). The homogenate was centrifuged (960 ×*g*, 10 min, 4 °C) and the supernatant was removed and 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, and the eluate collected. Each fraction was evaporated with ethylacetate, and the dry residue redissolved in ethanol. PGE_2 was determined by a competitive enzyme immunoassay (ELISA) kit (Assay Designs, Inc.). PGE_2 levels were quantified as PGE_2/mg tissue.

2.9. Isolation of cytoplasmic and nuclear proteins and immunoblotting detection

Nuclear proteins were isolated by the method of Helenius et al. (1996). Frozen colonic tissues were weighed and homogenized in ice-cold hypotonic buffer (1.5 mM $MgCl_2$, 10 Mm KCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1.0 mM dithiothreitol (DTT) and 10 mM Hepes, pH 7.9). Homogenates were incubated for 10 min on ice and centrifuged (25,000 ×*g*, 15 min, 4 °C). Cytoplasmic proteins were collected from the supernatants and nuclear proteins from the pellets. These were washed once and centrifuged at 10,000 ×*g*, 15 min, 4 °C) after which they were suspended in ice-cold low-salt buffer (25% v/v glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 1.0 mM DTT, KCl, Hepes, pH 7.9). Nuclear proteins were released by adding a high-salt buffer (25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 1.0 mM DTT, 1.2 M KCl, 20 mM Hepes, pH 7.9) drop by drop to a final concentration of 0.4 M KCl. Samples were incubated on ice for 30 min, with smooth shaking. Soluble nuclear proteins were recovered by centrifugation (25,000 ×*g*, 30 min, 4 °C) and proteins were stored at −80 °C.

Protein concentration of the homogenate was determined following Bradford colorimetric method (Bradford, 1976). Aliquots of supernatants containing equal amounts of protein (30–50 μg) were separated on 10% acrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In the next step, the proteins were electrophoretically transferred onto a nitrocellulose membrane and incubated with specific primary antibodies (Santa Cruz Biotechnology, CA) for COX-1 (M-20) at a dilution of 1:2000, COX-2 (M-19) at a dilution of 1:400, NF-κB p65 (A), at a dilution of 1:200 and p38 MAPK at a dilution of 1:1000 respectively. Each filter was washed three times for 15 min and incubated with the secondary horseradish peroxidase-linked anti-goat (for COX-1 and COX-2) or anti-rabbit immunoglobulin G (for NF-κB p65 and p38 MAPK) antibodies (Santa Cruz Biotechnology, CA). To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody (Santa Cruz Biotechnology, CA). Immunodetection was performed using enhanced chemiluminescence light-detecting kit (Amersham, Arlington Heights, IL). Densitometric data were studied following normalization to the control (house-keeping gene). The signals were analyzed and quantified by a Scientific Imaging System (KODAK 1D Image Analysis Software).

2.10. Apoptosis

Cytoplasmic DNA fragments, which are indicators of apoptosis, were measured with a DNA cell death detection ELISA PLUS KIT (Roche Diagnostics) according to the manufacturer's instructions. Results were expressed as absorbance $\times 10^3$/mg protein.

2.11. Data analysis

All values in the figures and text are expressed as arithmetic means±standard error (S.E.M.). 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), using Tukey–Kramer multiple comparisons test as post hoc 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

3.1. Protective effects of rosiglitazone in acute TNBS-induced colitis in rats

48 h after intracolonic administration of TNBS, rats showed postration, piloerection and hypomotility. Macroscopic inspection of the cecum, colon and rectum showed evidence of severe

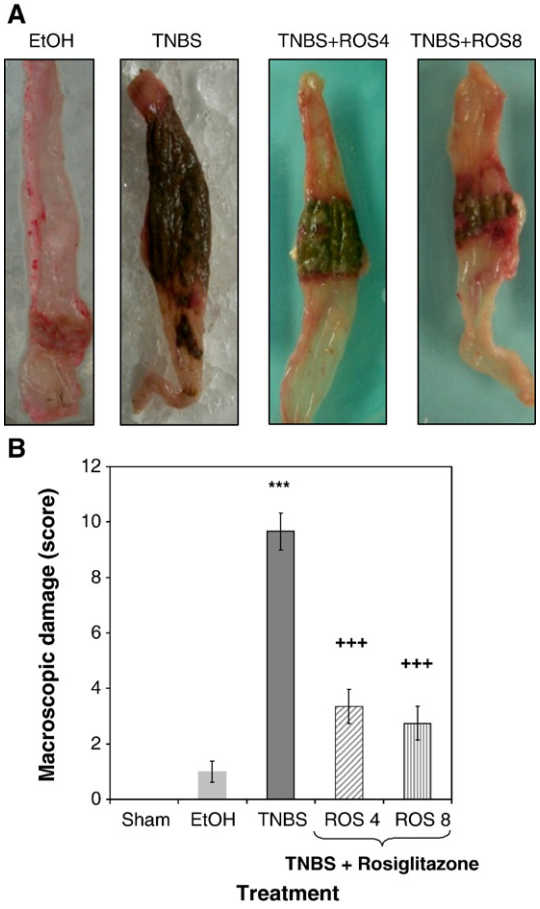

Fig. 1. (A) Macroscopic appearance of the colon of rats receiving vehicle only (EtOH), TNBS only or rosiglitazone treatment (4 or 8 mg/kg/day p.o.) 2 d before TNBS administration (rosiglitazone+TNBS). Rosiglitazone at the highest dose improved TNBS-induced colitis decreasing the presence of adhesions to adjacent organs and ulcerated surface. (B) Effects of acute administration of rosiglitazone on the colonic damage score. Colonic macroscopic damage resulting from TNBS (10 mg/animal) instilled into rat colon was scored as indicated in Section 2. Scores were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham, ethanol and TNBS groups), or in the presence of rosiglitazone (4 and 8 mg/kg/day p.o.). Data are expressed as mean±S.E.M. ***$P<0.001$ significantly different from sham. $^{++}P<0.001$ significantly different from TNBS.

colonic mucosal damage, with edema, deep ulcerations and haemorrhage (Fig. 1A). Lesions in the distal colon were quantified using a macroscopic damage score (mean: 9.66±0.66) (Fig. 1B). Control animals underwent severe anorexia with a marked body weight loss compared with the sham animals. A significant increase of weight/length of the rat colon, an indicator of inflammation, and presence of adhesions to adjacent organs were frequently observed in TNBS-treated rats (Table 1).

Treatment of TNBS-rats with rosiglitazone reduced the loss in body weight and the presence of adhesions to adjacent organs. There was a significant decrease in the weight/length relation of the rosiglitazone-treated rats' colon (Table 1) compared with TNBS-treated rats. In addition, TZD at the doses used 4 and 8 mg/kg p.o., significantly attenuated the extent and severity of the colonic injury (Fig. 1B). In fact, rosiglitazone was able to reduce the macroscopic damage score down to 2.74±0.6 ($P<0.001$) with the highest dose.

On histological examination of the colon from sham-treated rats, the histological features were typical of a normal structure (Figs. 2A,B and 3A). The histopathological features included transmural necrosis, edema and diffuse inflammatory cells (polymorphonuclear leukocytes, lymphocytes, and eosinophils) infiltration in the mucosa (Fig. 3B). We assessed focal ulceration of the colonic mucosa extending through the muscularis mucosae as well as desquamated areas or loss of the epithelium (Figs. 2C, D and 3B). The architecture of the crypts was distorted and the lamina propria was thickened in peripheral areas of distorted crypts, especially in basal areas (Figs. 2C and 3B). 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 had disappeared (Fig. 2D).

Rosiglitazone treatment caused an attenuation of morphological signs of cell damage and inflammatory cells were not found in lamina propria (Fig. 3C, D and E). In some areas, the epithelium remained intact (Fig. 2E) and the mucin layer was clearly visible with Alcian blue-positive cells; these observations suggest the beginning of a reepithelization and healing process in rosiglitazone-treated rats (Fig. 2F).

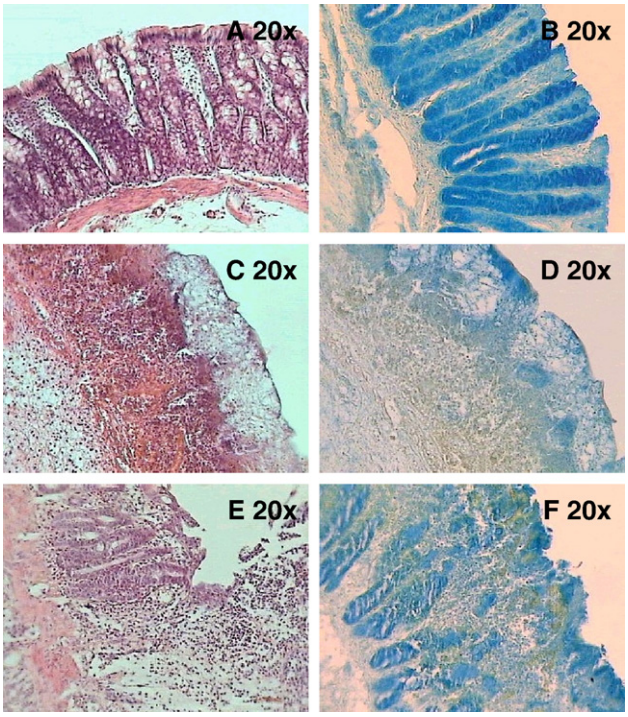

Fig. 2. Acute colitis model induced by TNBS: effect of rosiglitazone on colon injury. Histological appearance of rat colonic mucosa after haematoxylin and eosin stain (H–E), Alcian blue stain (AB), sham (A and B), and treated with TNBS 10 mg/animal (C and D), and rosiglitazone 8 mg/kg p.o. (E and F). (A) and (B) No histological modification was present in the sham animals. (C) and (D) 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. (E) Treatment with rosiglitazone 8 mg/kg p.o. reduced the morphological alterations associated with TNBS administration protecting the mucosal architecture. (F) Some areas showed accumulation of mucus and cell remnants; however, Alcian blue-positive cells were less numerous, and the mucin layer of the epithelium was missing. Original magnification 20×.

3.2. MPO, TNF-α and PGE_2 levels were significantly decreased after rosiglitazone administration

As shown in Table 2, a marked increase in MPO activity, an infiltration indicator of the colon with polymorphonuclear leukocytes also characterized the colitis caused by TNBS. This result was consistent with the histological findings. Treatment of TNBS-treated rats with the PPARγ agonist significantly ($P<0.01$) reduced the degree of polymorphonuclear neutrophil infiltration.

Colonic injury by TNBS administration was also characterized by an increase of the proinflammatory cytokine TNF-α (Table 2). In contrast, the levels of this cytokine were significantly lower ($P<0.05$ and $P<0.01$) in rats treated with rosiglitazone 4 and 8 mg/kg p.o., respectively.

Table 1
Quantified parameters after administration of rosiglitazone (4 and 8 mg/kg p.o.) in rats with acute colitis induced by TNBS intracolonic instillation (10 mg/animal)

Group	*n*	Body weight changes (g)	Adhesions (score 0–2)	Diarrhoea (score 0–1)	Colon weight/colon length (g/cm)
Sham	14	11.0±8.2	0	0	0.01±0.01
EtOH	14	1.4±2.9	0	0.2±0.2	0.02±0.02
TNBS	12	−37.77±30.53[a]	1.6±0.22[b]	1.4±0.52[b]	0.31±0.07[a]
TNBS+ROS 4	12	3.88±27.6[d]	0.8±0.25[c]	0.3±0.48[e]	0.21±0.05[d]
TNBS+ROS 8	12	25.71±16.18[e]	0.75±0.16[c]	0.5±0.53[e]	0.29±0.06[e]

Colonic parameters were quantified in the sham group ($n=14$), which received saline instillation. TNBS group ($n=14$) received TNBS intracolonically in a vehicle of 50% (v/v) ethanol; ethanol group ($n=14$) received 50% (v/v) ethanol intracolonic injection. Data are expressed as mean±S.E.M.
[a]$P<0.01$ and [b]$P<0.001$, significantly different from sham; [c]$P<0.05$, [d]$P<0.01$ and [e]$P<0.001$, significantly different from TNBS.

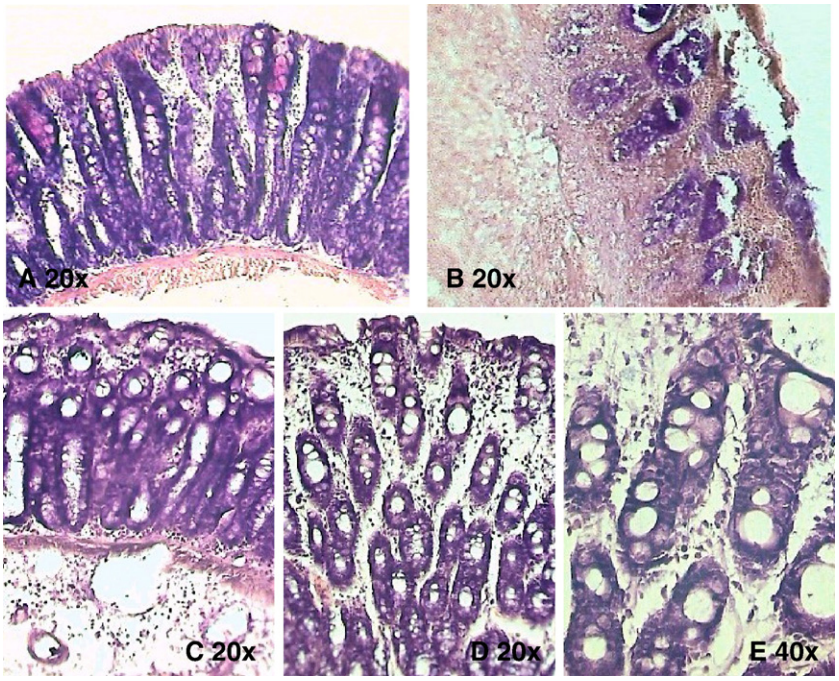

Fig. 3. Rat colon segments stained with Giemsa: sham (A), and treated with TNBS 10 mg/animal (B), and rosiglitazone 8 mg/kg p.o. (C, D and E). Infiltration of inflammatory cells was highly observed in the colonic mucosa of TNBS-treated animals. Rosiglitazone prevented development of inflammatory changes. Original magnifications 20× and 40×.

Our data also showed that PGE_2 content increased significantly ($P<0.001$) in colonic mucosa of TNBS group compared with sham animals. In addition, under our experimental conditions, the tested doses of rosiglitazone significantly reduced ($P<0.05$) the rise in the PGE_2 generation compared with TNBS group (Fig. 4) in both doses assessed.

3.3. Rosiglitazone seems to modulate the acute colitis through NF-κB p65 and p38 MAPK signalling pathways

The expression levels of cyclooxygenases, p38 MAPK, and NF-κB p 65 were measured by western blotting of cytosolic (Fig. 5A) and nuclear extracts (Fig. 5B), respectively, from colonic mucosa. NF-κB p65 protein was not detected in nuclei of normal colon mucosa whereas a high nuclear factor expression appeared in colon mucosa from control TNBS-treated rats. Nevertheless, upon treatment with PPAR-γ agonist, the protein expression of NF-κB p65 was drastically decreased. As shown in this figure, the levels of COX-1 protein remained unchanged in all groups; however, rosiglitazone induced downregulation of COX-2 in the treated

Table 2
Myeloperoxidase activity (MPO, U/mg tissue) and tumour necrosis factor alpha levels (TNF-α, pg/mg tissue) after rosiglitazone (4 and 8 mg/kg p.o. respectively) in rats with acute colitis produced by TNBS intracolonic instillation (10 mg/kg)

Group	*n*	MPO (U/mg tissue)	*n*	TNF-α (pg/mg tissue)
Sham	10	0.78±0.06	10	254.38±51.51
EtOH	6	0.89±0.05	6	573.68±77.5
TNBS	10	1.38±0.11[a]	10	635.68±66.8[a]
TNBS+ROS 4	10	0.95±0.06[c]	10	395.52±40.8[b]
TNBS+ROS 8	10	0.93±0.09[c]	10	321.29±48.9[c]

Colonic mucosal MPO activity (U/mg tissue) and TNF-α levels (pg/mg tissue) were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham ($n=10$), ethanol ($n=6$) and TNBS ($n=10$) groups), or in the presence of rosiglitazone (4 and 8 mg/kg/day p.o., $n=10$). Data are expressed as mean±S.E.M.
[a]$p<0.001$ significantly different from sham; [b]$p<0.05$ and [c]$p<0.01$ significantly different from TNBS.

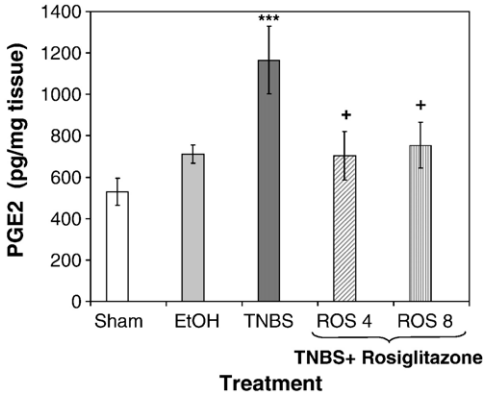

Fig. 4. Prostaglandin E_2 (PGE_2, pg/mg tissue) levels after rosiglitazone administration (4 and 8 mg/kg p.o.) in rats with acute colitis produced by TNBS intracolonic instillation (10 mg/animal). Prostanoids synthesis in the colonic tissue was quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham, ethanol and TNBS groups), or in the presence of rosiglitazone (4 and 8 mg/kg/day p.o.). Data are expressed as mean±S.E.M. ***$P<0.001$ significantly different from sham. $^{+}P<0.05$ significantly different from TNBS.

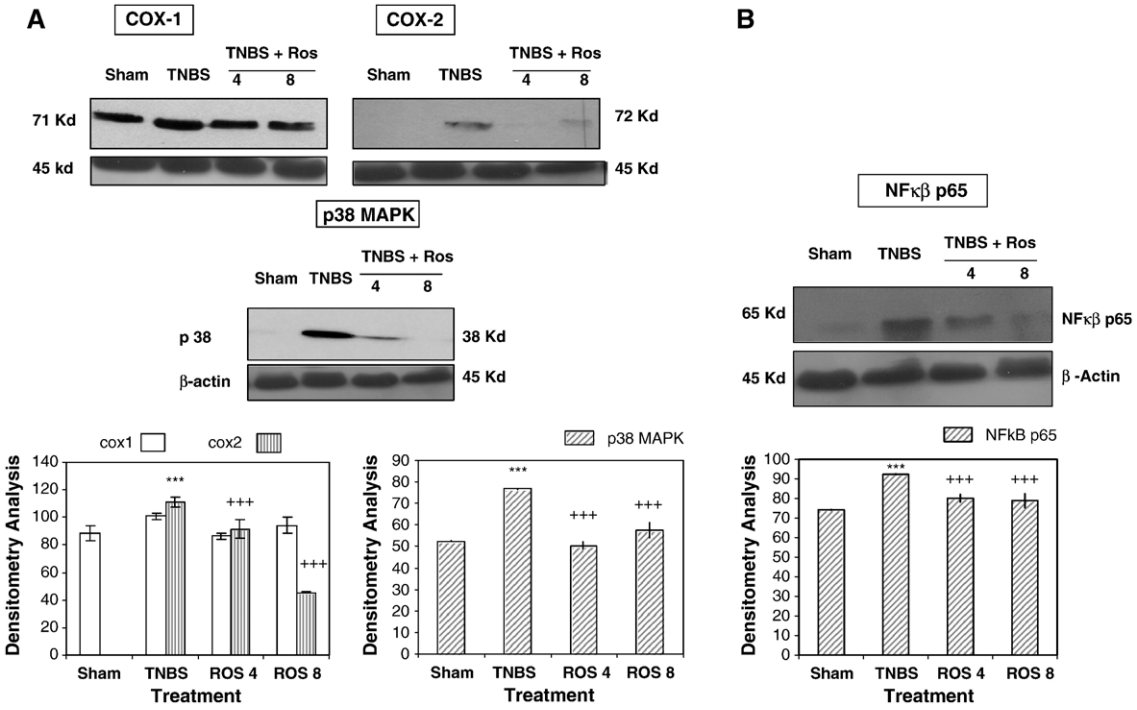

Fig. 5. Representative Western blot analysis comparing cytoplasmic (A) and nuclear (B) proteins. COX-1 protein remained unchanged in all groups (sham, TNBS and TNBS+Rosiglitazone (4 and 8 mg/kg p.o.)); however, rosiglitazone induced downregulation of COX-2 in the treated groups versus TNBS control. According to p38 MAPK protein was highly increased by TNBS; nevertheless, oral administration of rosiglitazone was able to diminish the upregulation of p38 MAPK protein (A). The protein expression of NF-κB p65 was drastically decreased in TNBS+rosiglitazone (4 and 8 mg/kg) groups p.o. Densitometric data were studied following normalization to the control (β-actin house-keeping gene). The results are representative of three experiments performed on different samples and data are expressed as mean±S.E.M. ***$P<0.001$ significantly different from sham. $^{+++}P<0.001$ significantly different from TNBS group.

groups versus TNBS control. On the other hand, p38 MAPK protein was highly increased by TNBS ($P<0.001$), indicating that the p38 MAPK protein expression could be induced at the acute stage of colonic lesion caused by TNBS. Nevertheless, oral administration of rosiglitazone was able to diminish the up-regulation of p38 MAPK ($P<0.001$).

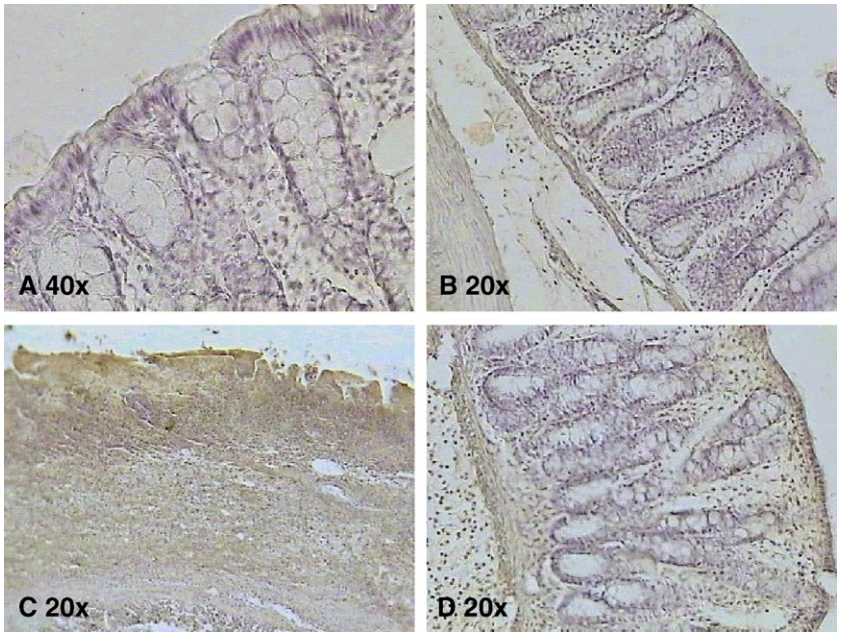

Fig. 6. Immunohistochemical localization of COX-1 isoenzyme in sections of colon. Negative control (A). In normal colon, colonocytes of the upper half of the crypts were found to be COX-1-positive (B). COX-1 immunosignal was weakly observed in the colon of TNBS-control rats (C). COX-1 expression of inflamed colon treated with rosiglitazone 8 mg/kg p.o. (D) reflected no important changes in relation to sham group. Original magnifications 20× and 40×.

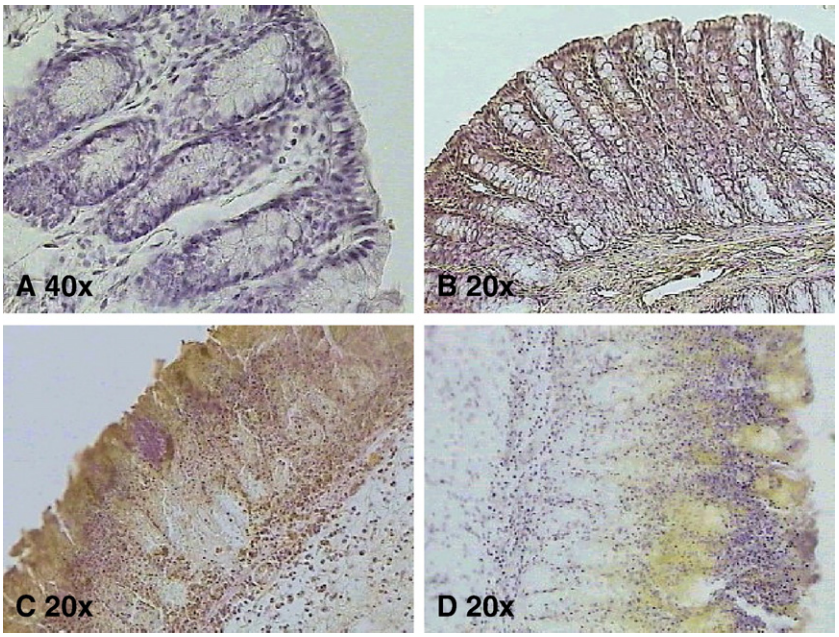

Fig. 7. Immunohistochemical localization of COX-2 isoenzyme in sections of colon. Negative control (A). COX-2 expression in normal colonic mucosa (B). COX-2 is strongly expressed in the colon of TNBS-control rats (C). COX-2 expression was decreased in apical epithelial cells of inflamed colon treated with rosiglitazone 8 mg/kg p.o. (D). Original magnifications 20× and 40×.

3.4. Immunohistochemical observations for COX-1 and -2 after rosiglitazone treatment

According to the immunohistochemical observations, in normal colons, specific immunosignals for COX-1 were obtained in surface epithelium as well as in the upper half of the crypts. Mononuclear cells of the lamina propria and the regional lymphatic nodules as well as cells of the muscularis mucosae showed COX-1 specific immunosignals (Fig. 6B) in relation with negative control (Fig. 6A). In the basal part of the crypts, COX-1 expression was restricted to individual cells, which according to morphological criteria are endocrine cells, a specialized epithelial cell type of the lower crypt. COX-2 specific immunolabelling was occasionally observed in colonocytes of the normal surface epithelium of matched control colon as shown in Fig. 7B.

Compared with normal colon, significant changes in the cellular distribution of COX-1 and COX-2 were observed in animals treated with TNBS in those colonocytes of the surface and the crypt epithelium, which were only weakly decorated by the COX-1 specific antiserum (Fig. 6C) whereas prominent COX-2 expression was found in cells of surface epithelium and the inflammatory infiltrate (Fig. 7C). COX-1 expression reflected no important differences in the cellular localization and the degree of positive staining for COX-1 in colon mucosa from rosiglitazone-treated rats after treatment (Fig. 6D). At this time, rosiglitazone-treated rats showed a lower level of expression of the inducible isoform in apical epithelial cells of inflamed colon (Fig. 7D).

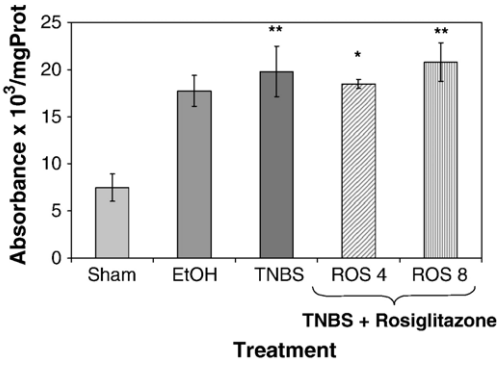

Fig. 8. Apoptosis observed in the colonic mucosa after acute colitis induced by TNBS (10 mg/animal). Apoptosis was quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham, ethanol and TNBS groups), or in the presence of rosiglitazone (4 and 8 mg/kg/day p.o.). Data are expressed as mean ± S.E.M. $^{+}P<0.05$ significantly different from sham. $^{++}P<0.01$ significantly different from sham.

3.5. Effects of rosiglitazone in apoptosis

To get a better understanding about the role of PPARγ agonist in apoptosis, we decided to evaluate its effects in colonic mucosa under early acute inflammatory conditions by an ELISA, which specifically detected cytoplasmic histone-associated DNA fragments, mononucleosomes, and oligonucleosomes. As shown in Fig. 8 apoptosis was observed in the colonic mucosa of sham animals. DNA fragmentation was dramatically increased in TNBS-treated rats. The treatment of rats with the PPARγ agonist (4 and 8 mg/kg p.o.) caused no significant increase in TNBS-induced apoptosis compared with TNBS group.

4. Discussion

In the present study we have demonstrated for first time that the PPARγ ligand, rosiglitazone, attenuated TNBS-induced

acute colonic injury in rats. There was an attenuation of morphological signs of cell damage, the colonic mucosa showed ulcers at the beginning of reepithelization and healing process. The decrease in the extent of colitis was accompanied by a lower body weight loss in the animals and a decrease of adhesions between the colon and adjacent organs. The presence of adhesions, which results from transmural inflammation is a common feature of TNBS colitis (Morris et al., 1989; Villegas et al., 2003a,b). The reduction in the incidence of adhesions suggests a beneficial effect of rosiglitazone on the extension of the inflammatory process in this experimental model.

Our results also revealed that rosiglitazone increased the amount of mucus stained by Alcian blue (acid glucoproteins such as sialomucins) in colon mucosa. Alcian blue-positive cells seem to be associated with regenerative processes of the mucosa (Alarcón de la Lastra et al., 1994; Martin et al., 2004). By contrast, its reduction has been related to a decreased resistance of the mucosa and paralleled by alterations in the normal pattern of maturation of the mucin in globet cells (Torres et al., 1999; Einerhand et al., 2002).

Infiltration of leukocytes into the mucosa plays a crucial role in the development of tissue necrosis and mucosal dysfunction associated with colitis as they represent a major source of reactive oxygen and nitrogen species in the inflamed colonic mucosa (Bell et al., 1995). Activated neutrophils produce superoxide anion, the main free radical in tissues, which through NADPH oxidase reduces molecular oxygen to the superoxide anion radical, and through the enzyme MPO which catalyzes the formation of such potent cytotoxic oxidants as hypochlorous acid from hydrogen peroxide and chloride ions and *N*-chloramines (Villegas et al., 2003a). It has been suggested that the main chemoattractants for neutrophils are pro-inflammatory cytokines, such as IL-1β, interferon-γ and TNF-α, that regulate endothelial molecule expression (ICAM-1) on vascular endothelial cells and promote neutrophil adherence to these cells (Villegas et al., 2003a,b; Deniz et al., 2004).

Despite the fact that the cytokine profiles associated with ulcerative colitis and Crohn's disease are classically considered different (a Th2 pattern in ulcerative colitis and a Th1 pattern in Crohn's disease) (Cottone et al., 2006), there is enough evidence *in vitro* and *in vivo* that TNF-α plays an important role in ulcerative colitis (Bouma and Strober, 2003). For example, reports from previous studies (Villegas et al., 2003a,b; Sanchez-Hidalgo et al., 2005; Martin et al., 2006) indicated that TNF-α production plays an important role in TNBS-induced colitis. Our findings, as in the above-cited papers, show that the MPO activity (an index of tissue-associated neutrophil accumulation) and the production of the proinflammatory cytokine TNF-α were correlated with the development of colonic inflammation. Thus, the ability of the PPARγ agonist to reduce the inflammatory cell infiltrate in the colon could partly explain the observed reduction in the cytokine levels and may contribute to the attenuation in the formation of reactive oxygen and nitrogen species. Similar results were obtained in a model of colitis under chronic experimental conditions (Sanchez-Hidalgo et al., 2005). Likewise, these results agree with those published *in vivo* data where rosiglitazone has been shown to reduce the levels of these inflammatory mediators (Villegas et al., 2004; Collino et al., 2006) although data from a recent study has suggested an additional anti-inflammatory mechanism for rosiglitazone through a PPARγ independent pathway in DSS-induced colitis in mice (Adachi et al., 2006).

In our study, we have demonstrated that the protective effect of rosiglitazone on early colonic inflammation involves inhibition of MAPK and NF-κB signalling pathways. Both mechanisms may be functionally interconnected and not act independently. Inhibition of the p38 MAPK pathway has been shown to abolish NF-κB-driven stimulation of gene expression (Carter et al., 1999) and p38 MAPK activation has been suggested to contribute to NF-κB stimulation by modulating the transactivation capacity of the NF-κB p65 subunit (Vanden Berghe et al., 1998). NF-κB has been shown to activate, via transcription of genes that encode pro-inflammatory cytokines (TNF-α, IL-1β, IL-12, and IL-6) in different cell-types, the expression of enzymes (e.g. iNOS and COX-2) and monocyte-chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), and intracellular adhesion molecule (ICAM), interfering with leukocyte chemo-attraction and cellular adhesion to endothelial cells (Dubuquoy et al., 2002).

In particular we found the presence of detectable quantities of NF-κB p65 in the nuclear extracts while the nuclear protein expression of NF-κB p65 was drastically decreased upon rosiglitazone treatment to rats. The p65 subunit activation has a significance in inflammatory bowel disease since it is highly activated in the mucosal biopsy specimens of patients with ulcerative colitis and Crohn's disease (Chung et al., 2000). The p65 antisense oligonucleotide treatment also aborts chronic intestinal inflammation in a murine model of inflammation (Lawrance et al., 2003). Our results agree with other authors which suggest that most anti-inflammatory properties of PPARγ agonists have been reached through NF-κB inhibition (Desreumaux et al., 2001; Daynes and Jones, 2002).

Our results also show that rosiglitazone can reduce p38 MAPK expression in colonic mucosa. There are three major groups of MAPKs in mammalian cells, the extracellular signal-related kinases (ERK), the c-Jun NH2-terminal kinases (JNK), and p38 MAPK, that are activated by phosphorylation. MAPK cascades transduce signals from the cell surface into changes in gene expression that control diverse functions such as inflammatory responses of epithelial cells (Jijon et al., 2002), proliferation, differentiation, transformation and apoptosis (Schlezinger et al., 2006). In particular, p38 MAPK is a key modulator of several target genes that ultimately control infiltration of monocytic cells, acute intestinal inflammation and intestinal electrolyte and water secretion, and in response to a variety of stimuli regulate cytokine production (Kyriakis and Avruch, 2001) and up regulates COX-2 expression in intestinal epithelial cells (Kim et al., 2005).

The importance of p38 MAPK in ulcerative colitis is supported by recent experiments where the use of p38 MAPK inhibitors abrogated colitis (Waetzig et al., 2002). Moreover, a recent study has demonstrated that it can be effective for human inflammatory bowel disease (Hommes et al., 2002). Recent

studies have clearly shown the implication of PPARγ agonists on the activation of MAPK cascades (Kim et al., 2003; Gardner et al., 2005). PPARγ agonists appear to act preferentially on p38 MAPK activation rather than on other subisoforms like p42/p44. Our results agree with a previous paper by Collino et al. in which it has been shown that rosiglitazone could inhibit both MAPK and NF-κB activation induced by cerebral ischemia/reperfusion (Collino et al., 2006).

The role of prostanoids in the intestinal inflammatory process is not totally understood. During the course of inflammatory bowel disease and experimental colitis some prostanoids are formed and negatively modulate the extension of the disease (Carty et al., 2000). Our results showed that the excessive production of PGE_2 and up-regulation of COX-2 were undoubtedly identified to be highly induced by TNBS. Administration of the PPARγ agonist, rosiglitazone, induced a significant reduction in colonic PGE_2 levels and a very weak COX-2-immunoreactivity. It is well known that this prostanoid is an essential inflammatory mediator that induces intestinal cell chloride and water secretion, which leads in diarrhoea observed in inflammatory bowel disease and body weight loss (Lanza et al., 1991). In addition, endogenous PGE_2 is produced by lamina propria mononuclear cells, dependent of COX-2 expression and is thought to be the most potent mediator of inflammation because of its 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-α and IL-1β (Newberry et al., 1999). Our data, which agree with previous studies (Guo et al., 2001; Kankuri et al., 2001; Martin et al., 2003, 2004; Kim et al., 2005), suggest that the increased PGE_2 production during TNBS-induced acute colitis is dependent upon the activity of COX-2 possibly via p38 MAPK and NFκB pathways. These observations suggest that the protective role of rosiglitazone in this experimental model is probably due to a mechanism dependent of PGE_2 synthesis by COX-2 through inhibition of the MAPK and NF-κB signalling pathways.

Apoptosis may represent a protective mechanism against neoplasic development in the organism by eliminating genetically damaged cells or excess cells that have improperly been induced to divide by a mitotic stimulus. However, deregulated apoptosis seems to be a major cause of the impaired barrier function, leading to the invasion of pathogenic microorganisms, to a leukocyte survival enhanced with exacerbation of the disease (Tardieu et al., 2000). Previous studies have shown significant apoptosis in colonic epithelial cells during mild acute inflammation induced by dextran sulphate sodium (Yue et al., 2001) and TNBS-induced colitis (Tardieu et al., 2000). These findings are in compliance with the present study, in which colonic cell death was associated with apoptosis in the colon lesion 48 h after intracolonic administration of TNBS. However, treatment of rats with rosiglitazone did not cause significant changes in TNBS-induced apoptosis.

An increase in apoptosis linked to PPARγ agonists has already been documented in *in vitro* studies, for instance, in several cancer cell lines (Chen et al., 2002; Contractor et al., 2005; Piva et al., 2005; Yang and Frucht, 2001; Li et al., 2003), and normal cells (Chinetti et al., 1998; Schlezinger et al., 2002, 2004). Mechanistic studies have further suggested that several TZDs, such as troglitazone and ciglitazone mediate apoptotic effects through a complexity of PPAR-independent mechanisms. Evidence indicates that PPARγ agonists might inhibit the antiapoptotic function of Bcl-xL/Bcl-2 by blocking BH3 domain-mediated heterodimerization with pro-apoptotic Bcl-2 members (Shiau et al., 2005). In addition, it has also been documented that p38 MAPK activation has been associated with PPARγ-induced apoptosis. For instance, a recent study by Kim et al. showed that the MAPK signalling pathways played an active role in mediating the ciglitazone-induced cell death of osteoblasts (Kim et al., 2006); similar results were obtained by Schlezinger et al. in B cells with the PPARγ agonist GW7845. However, in our study the apoptotic activity of rosiglitazone was not linked to p38 MAPK activation, although further investigation will be required to confirm this last mechanism.

Overexpression of COX-2 has recently emerged as an important determining agent of cell adhesion, angiogenesis and apoptosis (Kohno et al., 2005). Yang et al. showed that activation of PPAR pathway by ciglitazone induced apoptosis and inhibition of COX-2 expression in human colon cells HT-29. Similar data were reported by Li et al. Furthermore, it is well documented that NF-κB plays a pivotal role in regulating programmed cell death, being able to activate both pro-apoptotic and anti-apoptotic genes. Given the correlation between increased COX-2 expression and cell adhesion, angiogenesis and apoptosis, we suggest that induction of apoptosis by rosiglitazone could be mediated through down-regulation of COX-2.

Together, our data confirm that rosiglitazone exerts protective effects in acute experimental colitis. The anti-inflammatory effects seem to be related to impairment of neutrophil function, absence of up-regulation of TNF-α and increase of mucus production in colon mucosa. Rosiglitazone also reduced PGE_2 production and the overexpression of COX-2, NF-κB p65 and p38 MAPK. We conclude that the PPARγ agonist rosiglitazone is able to attenuate experimental colitis through a mechanism which involves inhibition of MAPK and NF-κB signalling pathways. We propose that this agent may have therapeutic implications for human inflammatory bowel disease.

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