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Curcumin, a *Curcuma longa* constituent, acts on MAPK p38 pathway modulating COX-2 and iNOS expression in chronic experimental colitis

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Abstract

Ulcerative colitis (UC) is a nonspecific inflammatory disorder characterized by oxidative and nitrosative stress, leucocyte infiltration and up-regulation of pro-inflammatory cytokines. Mitogen-activated protein kinases (MAPKs), such as the p38 and the c-Jun N-terminal kinase (JNK) modulate the transcription of many genes involved in the inflammatory process. Curcumin is a polyphenol derived from *Curcuma longa*, which is known to have anti-inflammatory activity. The aim of this study was to study the effects and mechanisms of action of curcumin, on chronic colitis in rats. Inflammation response was assessed by histology and myeloperoxidase activity (MPO). We determined the production of Th1 and Th2 cytokines and nitrites in colon mucosa, as well as the expression of inducible nitric oxide synthase (iNOS), cyclo-oxygenase(COX)-1 and-2 by western blotting and inmmunohistochemistry. Finally, we studied the involvement of MAPKs signaling in the protective effect of curcumin in chronic colonic inflammation. Curcumin (50–100 mg/kg/day) were administered by oral gavage 24 h after trinitrobenzensulfonic acid (TNBS) instillation, and daily during 2 weeks before sacrifice. Curcumin significantly attenuated the damage and caused substantial reductions of the rise in MPO activity and tumour necrosis factor alpha (TNF)-α. Also curcumine was able to reduce nitrites colonic levels and induced down-regulation of COX-2 and iNOS expression, and a reduction in the activation of p38 MAPK; however, no changes in the activation of JNK could be observed. In conclusion, we suggest that inhibition of p38 MAPK signaling by curcumin could explain the reduced COX-2 and iNOS immunosignals and the nitrite production in colonic mucosa reducing the development of chronic experimental colitis.

Keywords: Curcumin; Colitis; Neutrophils; Inducible nitric oxide synthase (iNOS); Cyclo-oxygenase (COX)-2; Mitogen-activated protein kinases (MAPKs)

1. Introduction

Inflammatory bowel disease (IBD), collectively referred to as Crohn's disease (CD) and ulcerative colitis (UC), is a nonspecific inflammatory disorder involving mainly the colonic mucosa and submucosa. Activated immune cells, primarily represented by neutrophils,

Abbreviations: CD, Crohn's disease; COX, Cyclo-oxygenase; CUR, Curcumin; HETAB, Hexadecyl-trimethylammonium bromide; IBD, Inflammatory bowel disease; IFN, Interferon; IL, Interleukin; JNK, c-Jun N-terminal kinase; MAPKs, Mitogen-activated protein kinases; MPO, Myeloperoxidase activity; NF-κB, Nuclear factor kappa B; iNOS, Inducible nitric oxide synthase; NO, Nitric oxide; TMB, 3,3′,5,5′-tetramethylbenzidine; TNBS, Trinitrobenzenesulfonic acid; TNF-α, Tumor necrosis factor alpha; UC, Ulcerative colitis.

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macrophages and cytotoxic T cells, play the aggressor role by attacking and destroying the intestinal barrier either 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, signaling and gene expression [1]. Furthermore, reactive oxygen species can activate diverse downstream signaling pathways, such as MAPKs or the nuclear factor NF-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 gamma (IFN-γ), IL-12, and IL-6) in different cell-types, the expression of receptors essential for neutrophils activation and chemotaxis and certain proteins, important determinants of colonic damage, i.e. cyclo-oxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) [2,3].

COX-2 is expressed as an early response to pro-inflammatory mediators and mitogen stimuli. In our previous studies we have observed that the increased prostaglandins production during chronic colitis is dependent upon the activity of COX-2 [4,5]. Excessive production of nitric oxide (NO) by iNOS in chronic colitis may be detrimental to the integrity of the mucosa based on the generation of reactive nitrogen species which causes cellular degeneration in various tissues, contributing to the development of intestinal damage. iNOS acts in synergy with COX-2 to promote the inflammatory reaction. Furthermore, both COX-2 and iNOS expressions are up-regulated by activated MAPKs in intestinal epithelial cells [6].

Curcumin is a polyphenol found in the dietary spice, extracted from dried rhizomes of the perennial herb turmeric (*Curcuma longa* Linn), a member of the ginger family. Curcumin is used as a spice to give the specific flavour and yellow colour to curry [7]. As a traditional medicine, turmeric has also been widely used for centuries to treat inflammatory disorders in its original countries such as arthritis, colitis and hepatitis [8]. There are recent reports which document that curcumin decreases the degree of inflammation associated with experimental colitis [9–12]. Based upon these data there is no doubt that the polyphenol plays an important role in anti-inflammatory responses in colon. However, there are many interesting questions regarding the therapeutic activity of curcumin in IBD. Thus, the aim of this study was to get a better understanding of the effects and mechanisms of action of curcumin, on the chronic injury caused by intracolonic administration of trinitrobenzensulfonic acid (TNBS) in the rat. Inflammatory response was assessed by histology and myeloperoxidase activity (MPO) was measured as an index of neutrophil infiltration in the mucosa. Th1 and Th2 cytokines production such as TNF-α and IL-10 were also carried out. We determined the production of NO in colonic mucosa as well as, the expression of iNOS, COX-1 and-2 by western blotting and immunohistochemistry. Finally, we studied the involvement of p38 MAPK and JNK signaling pathways in the protective effect of curcumin in chronic colonic inflammation.

2. Material and methods

2.1. Experimental animals

Male and female Wistar rats supplied by the Animal Services, Faculty of Medicine, University of Seville, Spain, and weighing 180–220 g, were placed singly in cages with wire-mesh floors at 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. They were randomly assigned to groups of 8–14 animals. The 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. [13]. Briefly, rats were lightly anesthetized with penthobarbital 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 anus verge. TNBS (Sigma Aldrich-Company Ltd., Spain) dissolved in 50% (v/v) ethanol was instilled into the colon through the cannula (30 mg in a volume of 0.25 ml) to induce chronic 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. Control groups were separated for comparison with TNBS/ethanol instillation: rats in the sham group received physiological saline, instead of TNBS solution. Curcumin (50–100 mg/kg; Sigma-Aldrich, Company Ltd. Spain) was emulsified in 0.9% saline solution and Tween 20%, and administered by oral route 24 h after TNBS instillation and daily during the 2 weeks before the sacrifice of the rats. The control group also received the vehicle solution by oral route. The animals were sacrificed, using an overdose of anaesthetic. The rats were checked daily for behaviour, body weight, and stool consistency.

Table 1
Quantified parameters after administration of curcumin (CUR, 50 or 100 mg/kg p.o.) in rats with chronic colitis induced by TNBS intracolonic instillation (30 mg/animal)

Group	*n*	Body weight changes (g)	Adhesions (score 0–3)	Diarrhoea (score 0–3)	Colon weight/ length (g/cm)
Sham	8	96.2±9.39	0	0	0.14±0.01
TNBS	14	47.14±11.8**	2.2±0.27***	0.86±0.14**	0.27±0.04
TNBS+CUR 50	14	83.8±6.4^{+}	1.3±0.2^{+}	0.12±0.12^{+}	0.19±0.02
TNBS+CUR 100	14	97.5±9.5^{++}	1±0.13^{+++}	0.17±0.16^{+}	0.19±0.01

Colonic parameters were quantified in the sham group ($n=8$), which received saline instillation. TNBS group ($n=14$) received trinitrobenzene sulphonic acid (TNBS) intracolonically in a vehicle of 50% (v/v) ethanol. Data are expressed as mean±S.E.M. (**) $p<0.01$ and (***) $p<0.001$ vs. sham and ($^{+}$) $p<0.05$, ($^{++}$) $p<0.01$ and ($^{+++}$) $p<0.001$, vs. TNBS group.

2.3. Assessment of colitis

Severity of colitis was evaluated by an independent observer who was blinded to the treatment. The distal 10 cm portion of the colon was removed and cut longitudinally for each animal, slightly cleaned in physiological saline to remove fecal residues and weighed. Macroscopic inflammation scores were assigned based on clinical features of the colon (score 0–10): 0 (no damage), 1 (focal hyperaemia), 2 (ulceration without hyperaemia or bowel wall thickening), 3 (ulceration with inflammation at 1 site), 4 (2 sites of ulceration and inflammation), 5 (major sites of inflammation>1 cm along the organ), 6–10 (mayor sites of inflammation>2 cm along the organ). 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. [14]. Pieces of inflamed colon were collected and frozen in liquid nitrogen for measurement of 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 increasing concentrations of ethanol, and embedded in paraffin. Thereafter, sections of tissue were cut at 5 mm on a rotary microtome (Leica Microsystems, Wetzlar, Germany), mounted on clean glass slides and dried overnight at 37 °C. The sections were cleared, hydrated, and stained with haematoxylin and eosin, and Alcian blue for histological evaluation of colonic damage 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 digitized using a Kodak D290 Zoom camera (Eastman Kodak Co., USA) and Motic1Images 2000 release 1.1 (MicroOptic Industrial Group CO., Ltd.; B1 Series System Microscopes). Analysis of the figures was carried out by Adobe1Photoshop1 Version 6.0 (Adobe Systems) image analysis program.

2.5. Immunohistochemical study

Sections (5 mm thick) were mounted on slides, cleaned, and hydrated. The sections were treated with a buffered blocking solution (3% bovine serum albumin in phosphate-buffered saline (PBS)) for 15 min. Then, the sections were co-incubated with primary antibodies for COX-1 and -2 (goat polyclonal; Santa Cruz Biotechnologies, CA) at a dilution of 1:400 in PBS v/v, at room temperature for 1 and 24 h respectively followed by washing with PBS and co-incubated with secondary antibody (1:500 in PBS v/v; Santa Cruz Biotechnology, CA), 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′-diaminobencidine solution in darkness, at room temperature for 10 min. The sections were washed with Tris–HCl, stained with haematoxylin according to standard protocols, mounted with glycerin and observed in an Olympus BH-2 microscope.

2.6. Assessment of leukocyte involvement

Myeloperoxidase (MPO) activity was assessed as an index of neutrophil infiltration according to the methods of Grisham et al. [15]. One sample from the distal colon was obtained from all animals. 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 50 mM PBS, pH=6.0, containing

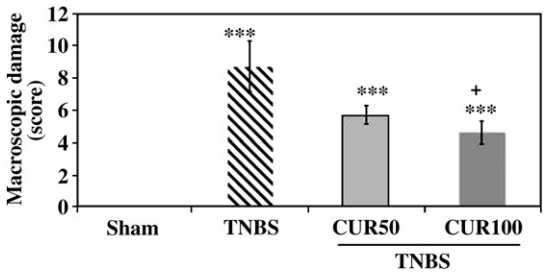

Fig. 1. Effects of chronic administration of curcumin on the colonic damage score. Colonic macroscopic damage resulting from trinitrobenzene sulphonic acid (TNBS, 30 mg/animal) instilled into rat colon was scored, as indicated in Section 2. Scores were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham and TNBS groups), or in the presence of curcumin (Cur: 50 or 100 mg/kg/day p.o,) Data are expressed as the mean±S.E.M. (***) $p<0.001$ vs. sham and (+) $p<0.05$ vs. TNBS group.

0.5% hexadecyl-trimethylammonium bromide (HETAB) and 10 mM 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 adding 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 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. Measurement of TNF-α and IL-10 production

Distal colon samples were weighed and homogenized, after thawing, in 0.3 ml PBS, pH 7.2 at 4 °C. They were centrifuged at 12,000 rpm for 10 min. Mucosal TNF-α and IL-10 levels were assayed with kit quantitative TNF-α and IL-10 enzyme

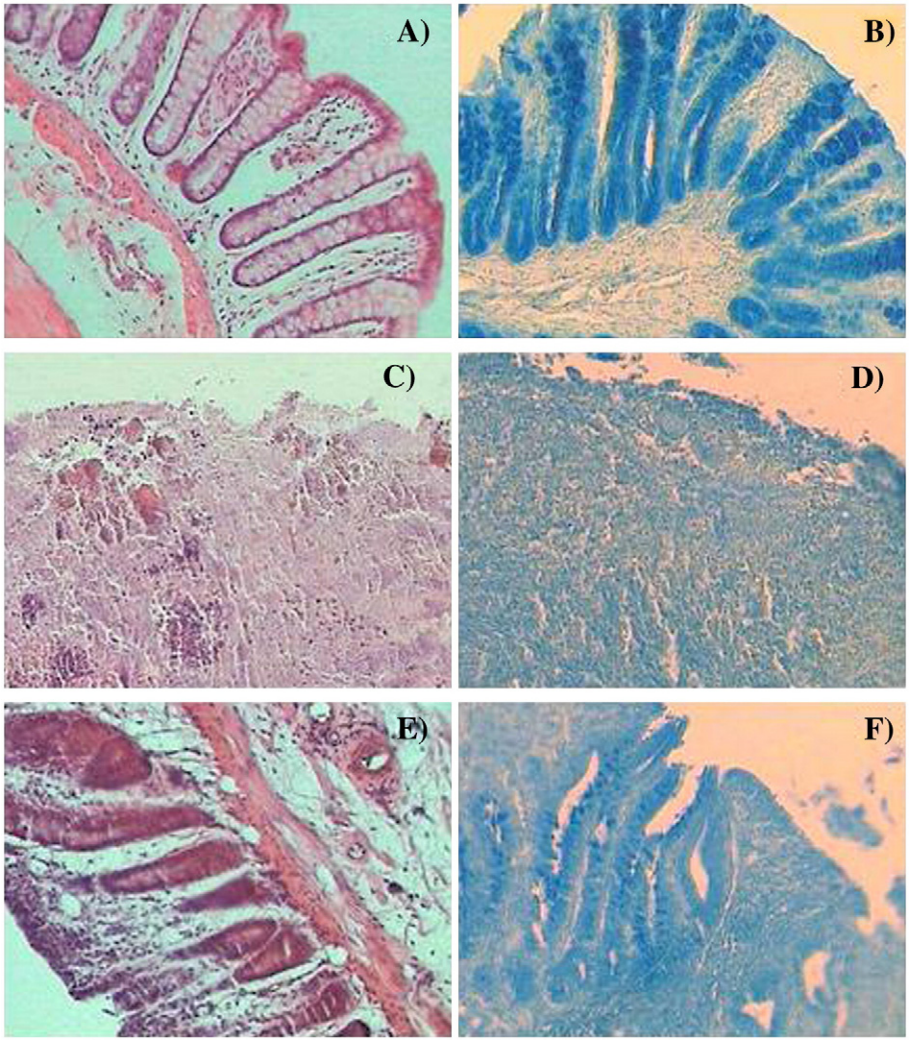

Fig. 2. Chronic colitis model induced by trinitrobenzene sulphonic acid (TNBS): effect of curcumin on colon injury. Histological appearance of rat colonic mucosa after hematoxylin and eosin stain (H–E), Alcian blue stain (AB): sham (A and B), and treated with TNBS 30 mg/animal (C and D), and curcumin 50 mg/kg p.o. (E and F). No histological modification was present in the sham animals (A and B). Mucosal injury was produced after TNBS administration, characterized by extensive granulation tissue with the presence of diffuse inflammatory infiltrates in the mucosa and submucosa (C and D). Treatment with curcumin 50 mg/kg reduced the morphological alterations associated with TNBS administration showing ulcers in the process of healing (E). 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 (F). Original magnification 200×.

Table 2
Myeloperoxidase activity (MPO, U/mg tissue), tumour necrosis factor alpha (TNF-α, pg/mg tissue) and interleukin 10 (IL-10) levels after administration of curcumin (CUR, 50 or 100 mg/kg p.o.) in rats with chronic colitis induced by TNBS intracolonic instillation (30 mg/animal)

Group	*n*	MPO (U/mg tissue)	TNF-α (pg/mg tissue)	IL-10 (pg/mg tissue)
Sham	8	5.1±1.1	2.3±0.8	16.9±1.9
TNBS	14	13.4±3.1***	9.2±0.7***	4.9±0.9***
TNBS+CUR 50	14	6.6±1^{+}	4.9±0.7^{+}	7.3±0.9^{+}
TNBS+CUR 100	14	5.2±1.2^{++}	2.7±0.4^{+++}	10.6±0.8^{+++}

Colonic mucosal MPO activity (U/mg tissue), TNF-α (pg/mg tissue) and IL-10 (pg/mg tissue) levels were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham and TNBS groups), or in the presence of curcumin (50 or 100 mg/kg/day p.o.) Data are expressed as the mean±S.E.M. (***) $p<0.001$ vs. sham and ($^{+}$) $p<0.05$, ($^{++}$) $p<0.01$ and ($^{+++}$) $p<0.001$ vs. TNBS group.

immunoassay kits (Diaclone, Besançon, France). The TNF-α and IL-10 values were expressed as pg/mg tissue.

2.8. Isolation of cytoplasmic proteins and western blot assay

Frozen colonic tissues were weighed and homogenized in ice-cold buffer (50 mM Tris–HCl, pH 7.5, 8 mM MgCl2, 5 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA), 0.5 mM EDTA, 0.01 mg/ml leupeptin, 0.01 mg/ml pepstatin, 0.01 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 250 mM NaCl). Homogenates were centrifuged (12,000 g, 15 min, 4 °C) and the supernatants were collected and stored at −80 °C. Protein concentration of the homogenate was determined following Bradford's colorimetric method. Aliquots of supernatants containing equal amounts of protein (30 μg) were separated on 10% acrilamide gel by sodium dodecyl sulfatepolyacryamide gel electrophoresis. In the next step, the proteins were electrophoretically transferred onto a nitrocellulose membrane (Potran, Schleicher and Schvell), as described by Towbin et al. [16], and incubated with specific primary antibodies for COX-1 (1:2000), COX-2 (1:400), p38 MAPK (1:1000), phospho p38 MAPK (1:1000); JNK (1:1000), phospho JNK (1: 1250) (all them from Santa Cruz Biotechnology, CA, California, USA) and iNOS (1:1000; Cayman, MI). Each filter was washed three times for 15 min and incubated with the secondary horseradish peroxidase linked anti-goat IgG (for COX-1 and-2), anti-rabbit IgG (for P-p38 and p38 MAPK and iNOS) and anti-mouse IgG (for JNK and P-JNK) antibodies (Santa Cruz Biotechnology, CA, California, USA). To prove equal loading, the blots were analysed for β-actin expression using an anti-β-actin antibody (Santa Cruz Biotechnology, CA, California, USA). Immunodetection was performed using enhanced chemiluminiscence light-detecting kit (Amersham, Arlinghton Heights, IL). Densitometric data were studied following normalisation to the control (house-keeping gene). The signals were analyzed

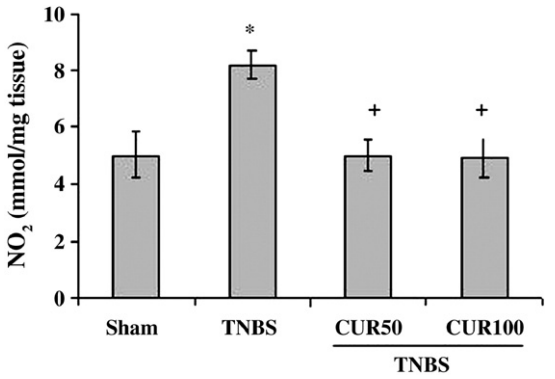

Fig. 3. Effects of chronic administration of curcumin on nitrite production in rats with colitis induced by trinitrobenzene sulphonic acid (TNBS, 30 mg/animal). Nitrite production was quantified in the absence of treatment, but with daily administration of the vehicle saline solution (TNBS group), or in the presence of curcumin (CUR, 50 or 100 μg/kg/day p.o.). The sham group received physiological saline instead of the TNBS solution in an equal volume. Data are expressed as the mean±S.E.M. (*) $p<0.05$ vs. sham and (+) $p<0.05$ vs. TNBS group.

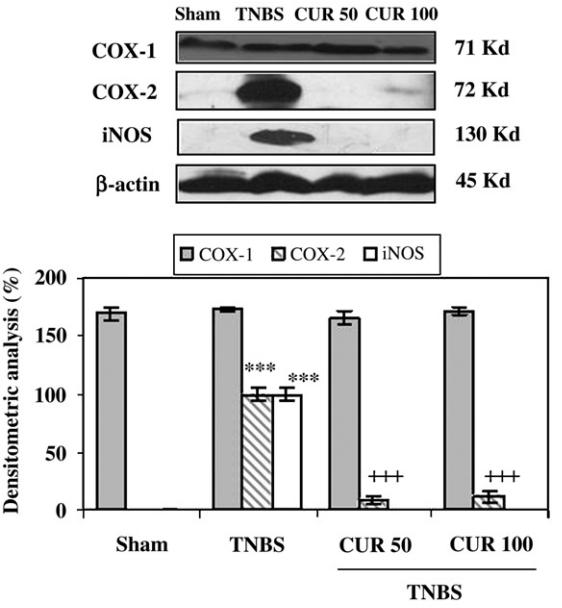

Fig. 4. Representative western blot analysis comparing cyclooxygenases (COX) and inducible nitric oxide synthase (iNOS). COX-1 protein remained unchanged in all groups. Chronic administration of curcumin (CUR, 50 or 100 mg/kg p.o.) induced down-regulation of COX-2 in the treated groups vs. TNBS control. The protein expression of iNOS was also decreased in TNBS+Curcumin groups. Densitometric data were studied following normalisation to the control (house-keeping gene). The results are representative of three experiments performed on different samples and data are expressed as the mean±S.E.M. (***) $p<0.001$ vs. sham and (+++) $p<0.001$ vs. TNBS group.

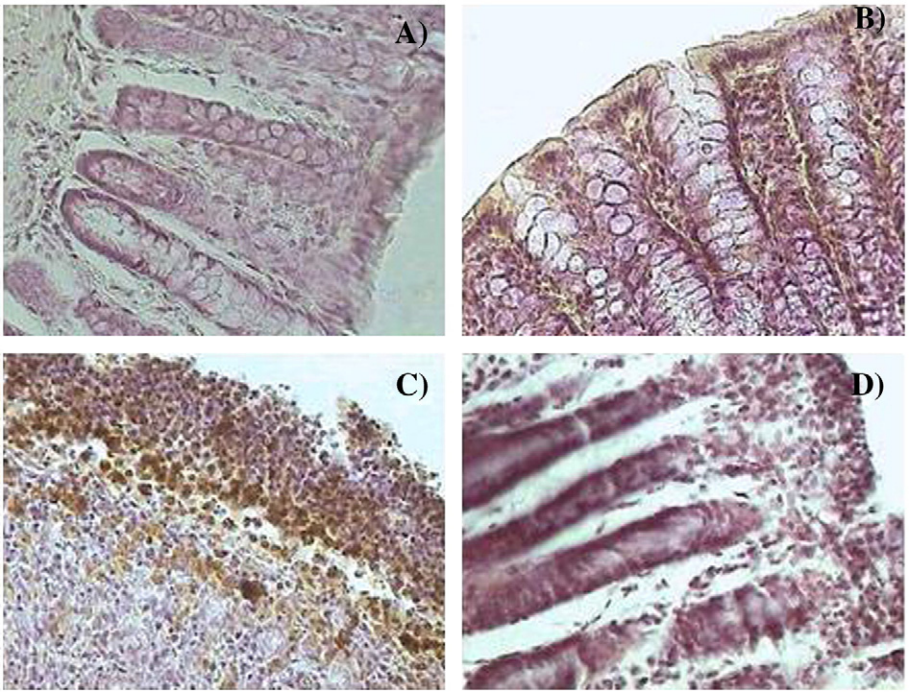

Fig. 5. Immunohistochemical localization of COX-2 isoenzyme in sections of colon. Negative control (A). COX-2 expression in normal colonic mucosa (B). COX-2 is strongly expressed in the colon of TNBS control rats (C). COX-2 expression was decreased in apical epithelial cells of inflamed colon treated with curcumin 50 mg/kg (D). Original magnifications 200×.

and quantified by a Scientific Imaging Systems (KODAK 1D Image Analysis Software).

2.9. Assessment on NO production

Distal colon samples were weighed and homogenized, after thawing, in 0.3 ml PBS, pH 7.2 at 4 °C. They were centrifuged at 12,000 rpm for 10 min. Nitrate was reduced to nitrite by incubation with nitrate reductase (670 mU/mL) and NADPH (160 mM) at room temperature for 3 h [10]. Nitrite+nitrate production, an indicator of NO synthesis, was measured using Griess reagent (1% sulphanilamide and 0.1% *N*-(1-napthyl)-ethylenediamine dihydrochloride in 5% H_3PO_4). 100 μL of the sample was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. Absorbance at 540 nm was then measured. The amount of nitrite released was quantified by comparison with sodium nitrite as standard.

2.10. Statistical evaluation

All values in the figures and text are expressed as arithmetic mean±standard error of the mean (S.E.M.). The 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 Tukey test. The Mann–Whitney *U*-test was chosen for non-parametric values. *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

As shown in Table 1, body weights of positive model rats, with TNBS-induced colitis, had obviously decreased and colitis gave rise to diarrhoea in the majority of animals. The inflammatory changes of the intestinal tract were associated with a significant augment ($p<0.01$) of weight/length of the rat colon, as an indicator of inflammation, and presence of adhesions to adjacent organs. Macroscopic inspection of the colon showed a flaccid appearance and evidence of bowel wall thickening, inflammation and ulcers. Lesions in the distal colon were quantified using a macroscopic damage score (mean: 8.7±1.5) (Fig. 1).

Curcumin treatment in TNBS-rats significantly reduced the loss in body weight and the presence of adhesions to adjacent organs. No significant increase in the weight/length of the rat colon was found in TNBS-rats, which had been treated with curcumin (Table 1). In addition, curcumin at the doses used 50 and 100 mg/kg p.o., significantly attenuated the extent and severity of the injury (Fig. 1), as evidenced by macroscopic damage score 5.7±0.5 and 4.6±0.6 respectively ($p<0.001$ vs. TNBS group).

On histological examination of the colon from sham-treated rats, the histological features of the colon were typical

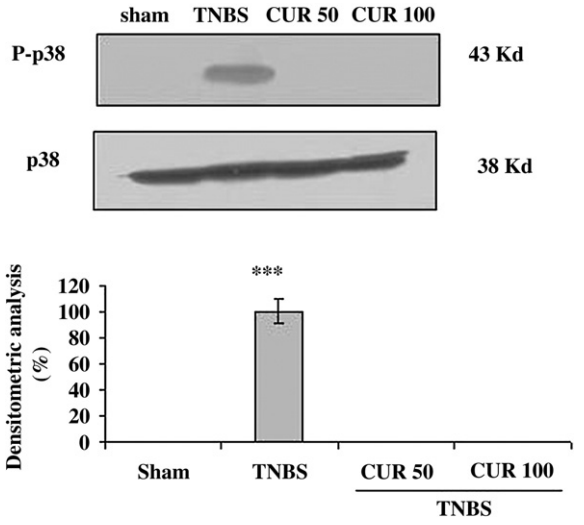

Fig. 6. Representative western blot analysis of expression and activation of the p38 MAPK using phosphospecific MAPK antibodies. p38 remained unchanged in all groups, however its activated form is up-regulated in TNBS group and diminished after chronic administration of curcumin (CUR, 50 or 100 mg/kg p.o.) The results are representative of three experiments performed on different samples and data are expressed as the mean±S.E.M. (***) $p<0.001$ vs. sham.

of a normal structure (Fig. 2A and B). In TNBS-treated rats, the inflammation extended through the mucosa, muscularis mucosae and submucosa. Extensive granulation tissue with presence of fibroblasts and lymphocytes, leukocytes, and diffuse inflammatory infiltrates was apparent. In some sections of ulcerated areas necrotic tissue adjacent to surface cells could be observed. The mucosa adjacent to ulcers showed grossly elongated crypts. Goblet cells were totally absent at the epithelium surface (Fig. 2C and D) compared to sham-treated rats (Fig. 2A and B). After administration of curcumin, the colonic histopathology was dramatically reduced: there was an attenuation of morphological signs of cell damage, the colonic mucosa showed ulcers in the process of healing, evolution to a more chronic inflammatory infiltrate, with mononuclear predominance and initiation of a repair process (Fig. 2E). Goblet cells with Alcian blue positive cells (acid glucoproteins such as sialomucins) were clearly observed in regions with reepithelization of the mucosal layer, in contrast, a remarkable mucin depletion was observed in ulcerative areas (Fig. 2F).

As show in Table 2, an important increase in MPO activity, an established marker for inflammatory cell infiltration (mainly polymorphonuclear leukocytes), also characterized the colitis caused by TNBS ($p<0.001$ vs. sham group), which was consistent with the histological findings. Moreover, after treatment with curcumin, data clearly indicated a significant reduction in this parameter ($p<0.05$ and $p<0.01$ respectively, vs. TNBS group). Colonic injury by TNBS administration was also characterized by an increase of the pro-inflammatory Th1 cytokine TNF-α and a diminution of the anti-inflammatory Th2 cytokine IL-10 ($p<0.001$ vs. sham group). In contrast, the levels of these cytokines were modified after curcumin treatment, TNF-α values were significantly lower and the IL-10 production was augmented in rats treated with curcumin compared with TNBS control rats (Table 2).

At 14 days after TNBS treatment, nitrite production was significantly elevated ($p<0.05$) compared to sham. However, curcumin treatment at dose levels of 50 and 100 resulted in a significant ($p<0.05$) decrease of its elevated production in the colon of TNBS-treated rats (Fig. 3).

The levels of expression of COX and iNOS were measured by western blotting of cytosolic extracts from colonic mucosa (Fig. 4). As shown in this figure, the levels of COX-1 protein remained unchanged in all groups. Exposure of colon to TNBS caused strong expression of COX-2 and iNOS, on the contrary curcumin induced down-regulation of COX-2 and iNOS in the treated groups versus TNBS control ($p<0.001$).

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 of slides of TNBS group showed COX-1 specific immunosignals (data not shown). On the contrary, COX-2 specific immunolabelling was scarcely found in the surface epithelium and mononuclear cells of lamina propria of mucosa in the sham group (Fig. 5B), whereas this expression was found elevated in cells of surface epithelium and in cells of the inflammatory infiltrate in the TNBS group (Fig. 5C). At this same time, curcumin-treated rats showed a lower level of expression of the inducible isoform in apical epithelial cells of inflamed colon (Fig. 5D).

We also examined the expression and activation of the p38 MAPK and JNK by western blot analysis using phosphospecific MAPK antibodies. To standardize protein loading in each line, blots were stripped and re-probed with the corresponding antibodies against both proteins. Intracolonic administration of

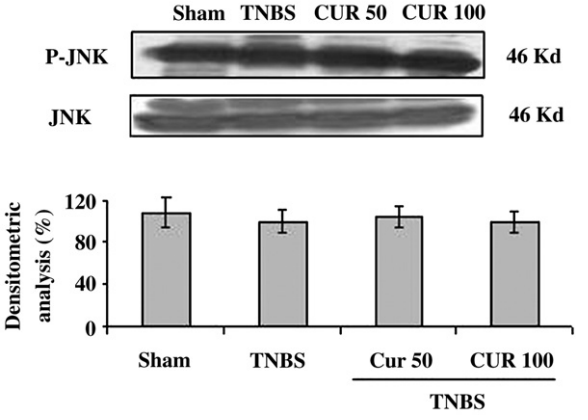

Fig. 7. Representative western blot analysis of expression and activation of the JNK using phosphospecific MAPK antibodies. JNK and its activated form remained unchanged in TNBS group and after chronic administration of curcumin (CUR, 50 or 100 mg/kg p.o.) The results are representative of three experiments performed on different samples and data are expressed as the mean±S.E.M.

TNBS resulted in a significant increase in the phosphorylation of p38 MAPK protein ($p<0.001$), indicating that the p38 MAPK protein activation could be induced at the chronic stage of colonic lesion caused by TNBS. Interestingly, administration of curcumin was able to diminish the activation of p38 MAPK ($p<0.05$) (Fig. 6). Nevertheless, no changes in the activation of JNK could be observed (Fig. 7).

4. Discussion

Our results demonstrate an improvement of TNBS-induced colitis in rats treated with curcumin as reflected in the experimental data, and by means of a macroscopic and histological disease score. Curcumin significantly ameliorated the appearance of diarrhoea and the disruption of colonic architecture. Moreover, there was an attenuation of morphological signs of cell damage, the colonic mucosa showed ulcers in the process of healing, and an evolution to a more chronic inflammatory infiltrate, with mononuclear predominance and initiation of a repair process. These results are consistent with previous data using different models of colonic models and dosages which document that curcumin is capable of decreasing the degree of inflammation associated with experimental colitis [9,10,11,12].

The protective effect of mucus as an active barrier may be attributed largely to its viscous and gel-forming properties that are derived from mucin glycoprotein constituents. In a previous paper, our results revealed that Alcian blue positive cells seem to be associated with regenerative processes of the colon mucosa [4,17,18]; 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 mucin in goblet cells [19]. Although bioavailability of curcumin is low after oral ingestion [20], it has been suggested that luminal curcumin may have a topical activity on colonic epithelial cells independent of systemic absorption. All these observations may have significance on the beneficial effect of curcumin in UC.

TNBS-induced colitis is an experimental model which mimics human CD, showing high amounts of Th1 cytokines (TNF-α, IL-12, IFN-γ, IL-1) but low levels of Th2 cytokines (IL-4, IL-5, IL-10) [21]. This model is also associated with an important inflammatory infiltration of lymphocytes and polymorphonuclear cells into the colon. Neutrophils represent a major source of reactive oxygen and nitrogen species in the inflamed colonic mucosa [4,21–23]. Reactive oxygen species and peroxynitrites induce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage. Activated neutrophils play a crucial role in the destruction of foreign antigens and in the breakdown and remodelling of injured tissue [24]. In addition, neutrophils can also release proteases, lactoferrin [25] and lipid mediators that can contribute to gastric injury.

It has been suggested that the main chemoattractants for neutrophils are pro-inflammatory cytokines, such as IL-1β, IFN-γ, and TNF-α that regulate endothelial molecule expression on vascular endothelial cells and promote neutrophil adherence to these cells. Reports from previous studies [4,5,18,23] indicated that TNF-α plays an important role in TNBS-induced chronic colitis and it likely is the regulator key of the inflammatory cascade in IBD, although data from other investigations suggested a controversial role for TNF-α in this pathology [26,27]. On the other hand, it has been reported that TNF-α signaling is linked to activated p38 MAPK. This cytokine is one of the best characterized agonists of the p38 and JNK pathways, itself regulated by p38 [3]. Our results show that MPO activity and pro-inflammatory cytokine TNF-α production were correlated with the development of colonic inflammation and moreover curcumin administration was able to diminish both parameters. These findings are supported by previous *in vivo* and *in vitro* experiments where curcumin has been shown to reduce the levels of these inflammatory mediators [9,10,28,29].

In addition, the degree of inflammation and damage induced by TNBS was paralleled to low levels of the anti-inflammatory cytokine IL-10 in colonic specimens. These data are in line with a previous study by Jian et al. [12] who documented a down-regulation of this cytokine in experimental UC, and interestingly the levels of this cytokine were significantly higher in the animals which had been treated with curcumin.

Our results also show that curcumin reduced P-p38 MAPK but it was not able to inhibit JNK activation in colonic mucosa. 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. They also regulate cytokine production [30] in response to a variety of stimuli and up-regulate COX-2 expression in intestinal epithelial cells [6]. The importance of p38 MAPK in UC is supported by recent experiments where the use of p38 MAPK inhibitors abrogated colitis [3]. Moreover, a recent study has demonstrated that it can be effective for human IBD [31]. Our results are in agreement with a previous study by Salh et al. [32] in which it has been shown that curcumin could inhibit p38 MAPK activation induced in acute experimental colitis.

In addition, our data confirmed that intracolonic TNBS did not alter the phosphorylation of JNK in

response to colonic injury. These results suggest that this MAPK is not involved in TNBS-induced chronic colitis. Indeed, treatment with curcumin did not modify JNK expression in comparison to controls. These findings are in accordance with Chen and Tan [33] who observed that curcumin was able to inhibit the JNK signaling pathway in *vitro*, but this inhibition could not fully account for the JNK inhibition by curcumin *in vivo*.

In the present study, we have also demonstrated that (1) macroscopic damage is associated with a high expression of COX-2, (2) iNOS expression is increased, (3) its high expression in colon is associated also with high levels of nitrites, (4) curcumin treatment reduced COX-2 and iNOS expression and returned nitrites to basal levels.

We have recently reported that there was a strong positive correlation between colitis score and labelling index of intestinal COX-2 expression [4,21–23] suggesting that the increased prostaglandin production during TNBS-induced acute colitis is dependent upon the activity of COX-2. Our results are also consistent with the findings of Tunstall et al. [34], who observed a down-regulation of the transcription of COX-2 in intestinal adenoma tissue from Apc(Min+) mice by curcumin. Furthermore, in *in vitro* studies curcumin inhibited COX-2 transcription in bile acid- and phorbol ester-treated human gastrointestinal epithelial cells [35], in macrophage-like differentiated RAW 264.7 cells [36] and in HT-29 human colon cancer cells [37]. Likewise, prolonged production of high amounts of NO by iNOS seems to enhance the inflammatory response and tissue injury in experimental colitis. Enhanced NO generation as well as high expression of iNOS in the inflamed colonic segments may be attributed to the contribution of macrophages and inflammatory neutrophils since NO production has also been established to be stimulated by lipopolysaccharide (LPS) and IFN-γ [38]. The present data are in agreement with those of Ukil et al. [10], who observed an increase in NO and iNOS expression compared to basal levels during the healing period of the colonic injury and a significant reduction after administration of curcumin in acute experimental colitis.

In accordance with reports in the literature, both COX-2 and iNOS enzymes expressions are up-regulated by activated MAPKs in intestinal epithelial cells [6]. Extensive research during the last few years has shown that curcumin mediates its anti-inflammatory effects through inhibiting the activation of the nuclear transcription factor (NF-kB). NF-kB activation is regulated by MAPKs through multiple mechanisms [39], and although the underlying mechanism of this suppressing action is still a matter of controversy, a possible mechanism might be the preventing of the NF-κB transactivation.

In conclusion, curcumin reduces the development of chronic experimental colitis and alleviates the inflammatory response. We suggest that inhibition of p38 MAPK signaling by curcumin could explain the reduced COX-2 and iNOS immunosignals as well as the decreased nitrite production in colonic mucosa.

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