

The intestinal anti-inflammatory effect of quercitrin is associated with an inhibition in iNOS expression

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1 Quercitrin, 3-rhamnosylquercetin, is a bioflavonoid with antioxidant properties, which exerts anti-inflammatory activity in experimental colitis. In the present study, different *in vivo* experiments were performed in order to evaluate the mechanisms of action involved in this effect, with special attention to its effects on proinflammatory mediators, including nitric oxide (NO).

2 Experimental colitis was induced in female Wistar rats by incorporation of dextran sodium sulfate (DSS) in drinking water. Oral treatment of quercitrin (1 or 5 mg kg⁻¹ day⁻¹) to colitic rats ameliorated the evolution of the inflammatory process induced when administered in a preventative dosing protocol. When quercitrin (1 mg kg⁻¹ day⁻¹) was administered on established colitis, it facilitated the recovery of the inflamed mucosa.

3 The beneficial effects exerted by quercitrin were evidenced both histologically and biochemically, and were associated with an improvement in the colonic oxidative status, altered as a consequence of the colonic insult induced by DSS. In addition, a reduction of colonic NO synthase activity was observed, probably related to a decreased expression in the inducible form of the enzyme *via* downregulation in the colonic activity of the nuclear factor- κ B.

4 Immunohistochemical studies showed that quercitrin treatment reduced macrophage and granulocyte infiltration in the inflamed tissue.

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Abbreviations: AP, alkaline phosphatase; DAI, disease activity index; DSS, dextran sodium sulfate; eNOS, endothelial nitric oxide synthase; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; LTB₄, leukotriene B₄; MPO, myeloperoxidase; NF- κ B, nuclear factor- κ B; NO, nitric oxide; NOS, nitric oxide synthase; TBS, Tris-buffered saline; TBSD-T, Tris-buffered saline-Tween-20; TNBS, trinitrobenzene sulphonic acid; TNF α , tumor necrosis factor α

Introduction

Chronic inflammatory bowel disease (IBD), mainly ulcerative colitis and Crohn's disease, is a naturally remitting and recurring condition of the digestive tract. Although its precise etiology is unknown, it is probably related to an abnormal exacerbated immune response to otherwise innocuous stimuli, which is not properly abrogated by the feedback system that normally downregulates the mucosal response to luminal factors (Fiocchi, 1998). As other inflammatory processes, IBD is characterized by an upregulation of the synthesis and release of a variety of proinflammatory mediators, such as eicosanoids, platelet-activating factor, reactive oxygen and nitrogen metabolites and cytokines, thus influencing mucosal integrity and leading to excessive tissue injury (Katz *et al.*, 1999; Podolsky & Fiocchi, 2000). Moreover, most of these mediators can induce the biosynthesis and release of other such

compounds, generating a 'vicious cycle' that may result in the propagation and perpetuation of the inflammatory response. Nowadays, a specific causal treatment of IBD is still not available, and the best strategy to effectively downregulate the exacerbated immune response that characterizes IBD may be to interfere with multiple stages of the inflammatory cascade, preferably with a single drug treatment (Kho *et al.*, 2001). In fact, the drugs currently used for the management of human IBD, that is, 5-aminosalicylic acid derivatives and systemic or local glucocorticoids, exert their beneficial effects through a combination of different mechanisms (Travis & Jewel, 1994; Bratts & Linden, 1996). Unfortunately, these drugs are not devoid of potentially serious side effects, thus limiting their use (Enns & Sutherland, 1998; Stein & Hanauer, 2000). For this reason, the development of new drug treatments that combine efficacy and safety is an important goal in IBD therapy.

This could be the case of flavonoids, natural products presenting several biological activities, mainly related to their ability to inhibit enzymes and/or their antioxidant properties, and which have also been described to downregulate the

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immune response (Middleton *et al.*, 2000). These activities could make their consideration as valid drugs in the pharmacological treatment of IBD. In fact, previous studies have shown the efficacy of some of these compounds, including quercitrin, rutoside, diosmin, hesperidin and morin, in the trinitrobenzene sulphonic acid (TNBS) model of rat colitis (Sanchez de Medina *et al.*, 1996; Cruz *et al.*, 1998; Crespo *et al.*, 1999; Galvez *et al.*, 2001). Although the mechanism of action of these flavonoids is poorly understood, a common feature in all cases is their ability to improve the colonic oxidative stress that characterizes the intestinal inflammatory status, probably related to their well-known antioxidant and/or free radical scavenging properties attributed to flavonoids. However, additional mechanisms might contribute in their beneficial effects. Morin has been shown to downregulate mediators involved in the intestinal inflammatory response, such as cytokines and nitric oxide (NO) (Galvez *et al.*, 2001). Recently, it has been reported that the beneficial effects of quercitrin on TNBS chronic colitis can arise from an early downregulation of the inflammatory cascade, which is associated with amelioration of the disturbances in hydroelectrolytic transport (Sanchez de Medina *et al.*, 2002).

The aim of the present study was to further elucidate the mechanisms involved in the intestinal anti-inflammatory effects of quercitrin. For this purpose, we tested the effects of this flavonoid in the dextran sodium sulfate (DSS) model of rat colitis in two different dosing protocols, that is, preventative and after an initial colonic insult. The DSS model produces inflammation limited to the colonic mucosa that is more closely related to human ulcerative colitis (Kullmann *et al.*, 2001; Gaudio *et al.*, 1999), as opposed to the TNBS model of rat colitis, which induces a transmural lesion with pathological characteristics similar to Crohn's disease (Fuss *et al.*, 1999). Special attention was paid to the effects exerted by the flavonoid on the production of some of the mediators involved in the inflammatory response: leukotriene B₄ (LTB₄) and NO.

Methods

Animals

Female Wistar rats (170–190 g) obtained from the Laboratory Animal Service of the University of Granada were housed individually in makrolon cages, maintained in air-conditioned animal quarters with a 12 h light–dark cycle, and fed standard rodent chow (Panlab A04, Panlab, Barcelona, Spain) and water *ad libitum* throughout the experiment. This study was carried out in accordance with the Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes of the European Union (86/609/EEC).

Induction of colitis and treatment protocols

After a 7-day acclimation period, rats were weighed and randomly distributed in the different experimental groups of eight rats each. Two experimental protocols were followed: (A) Preventative treatment, in which colitis was induced as described previously (Stucchi *et al.*, 2000) by replacing normal drinking water with distilled water containing 5% DSS (w v⁻¹, prepared daily, mol wt 36,000–50,000) for 8 days. The experimental groups were: a noncolitic group that received

no flavonoid treatment and distilled water without DSS, a colitic control group that received 5% DSS alone and the treated group that received 5% DSS and quercitrin (at doses of 1 and 5 mg kg⁻¹ day⁻¹). Quercitrin was dissolved in distilled water and administered orally by means of an esophageal catheter, beginning concurrently with DSS administration. All controls received equivalent volumes of water for the duration of the study. All animals were killed 8 days after the beginning of the experiments. (B) Quercitrin treatment on established colitis, in which colitis was induced as described previously, but DSS concentration was 5% (w v⁻¹) for the first 5 days and it was decreased to 2% (w v⁻¹) for the following 10 days. Similarly, three experimental groups of rats were included: noncolitic, control colitic and quercitrin treated; however, quercitrin treatment (at the dose of 1 mg kg⁻¹ day⁻¹) started when the concentration of DSS was reduced. All animals were killed after 15 days of the beginning of the experiments.

Assessment of colonic damage

Animal body weight, the presence of gross blood in the feces and stool consistency were recorded daily for each rat by an observer unaware of the treatment. These parameters were each assigned a score according to the criteria proposed by Cooper *et al.* (1993) (Table 1), which was used to calculate an average daily disease activity index (DAI) for each animal as these authors described previously. Food and water consumption was also recorded daily throughout the duration of the study. Once rats were killed by cervical dislocation, their colons were immediately removed and rinsed with ice-cold phosphate-buffered saline. The excised colonic segments were placed on an ice-cold plate, cleaned of fat and mesentery, and blotted on filter paper. Each specimen was weighed and its length measured under a constant load (2 g). The colon was longitudinally opened and a cross section from the distal diseased area was immediately fixed in 4% formaldehyde and embedded in paraffin for histological analysis.

The colon was subsequently sectioned in different fragments to be used for biochemical determinations, which were immediately frozen at –80°C, except for the samples used for glutathione content determination, which was immediately weighed and then frozen in 1 ml of 5% (w v⁻¹) trichloroacetic acid, and for LTB₄ synthesis determination, which was immediately processed.

Biochemical determinations in colonic tissue

All biochemical measurements were completed within 1 week from the time of sample collection and were performed in

Table 1 Scoring of disease activity index (DAI)

Score	Weight loss (%)	Stool consistency	Rectal bleeding
0	None	Normal	Normal
1	1–5		
2	5–10	Loose stools	
3	10–20		
4	>20	Diarrhea	Gross bleeding

DAI value is the combined scores of weight loss, stool consistency, and bleeding divided by 3. Adapted from Cooper *et al.* (1993).

duplicate. Myeloperoxidase (MPO) activity was measured according to the technique described by Krawisz *et al.* (1984); the results were expressed as MPO units ($\mu\text{mol min}^{-1}$) per gram of wet tissue. Total glutathione content was quantified with the recycling assay described by Anderson (1985), and the results were expressed as $\text{nmol g wet tissue}^{-1}$. Alkaline phosphatase (AP) activity was determined spectrophotometrically using disodium *p*-nitrophenylphosphate as substrate (Sanchez de Medina *et al.*, 1996) and the results expressed as $\text{mU mg protein}^{-1}$. Colonic nitric oxide synthase (NOS) activity was determined, in the presence or absence of aminoguanidine (10^{-4}M), by monitoring the conversion of L-[^3H]arginine to L-[^3H]citrulline as described previously (Rodriguez-Cabezas *et al.*, 2002), and the results were expressed as $\text{pmol L-citrulline } 30\text{ min}^{-1}\text{ mg protein}^{-1}$. Colonic samples for LTB_4 synthesis were minced on an ice-cold plate and suspended in a tube with 10 mM sodium phosphate buffer (pH 7.4) ($1/20\text{ w v}^{-1}$). The tubes were placed in a shaking water bath (37°C) for 20 min and centrifuged at $9000 \times g$ for 30 s at 4°C . The supernatants were frozen at -80°C until assay, and LTB_4 was quantified by enzyme-linked immunosorbent assay (ELISA) (Amersham, Madrid, Spain), and the results expressed as ng g^{-1} wet tissue.

Microwell colorimetric NF- κ B assay

Colonic NF- κ B activation was determined in colonic specimens obtained from those rats that received quercitrin once the colonic inflammation was in progress, and this was performed by an ELISA kit (TransAM™ method, Active Motif, Carlsbad, CA, U.S.A.). Colon homogenates were suspended in lysis buffer (20 mM HEPES (pH 7.5), 0.35 M NaCl, 20% glycerol, 1% NP-40, 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 mM EDTA, 0.1 mM EGTA) containing protease and phosphatase inhibitors (aprotinin, leupeptin, 1,10-phenanthroline, PMSF, ioadacetamide and sodium orthovanadate). After 10 min on ice, the lysate was centrifuged for 20 min at $12,000 \times g$, with the supernatants constituting the total protein extract being stored at -80°C until subjected to analysis. In all, $2\text{ }\mu\text{g}$ of each protein extract was incubated in a 96-well plate coated with immobilized oligonucleotide containing the NF- κ B consensus site ($5'$ -GGGACTTTC- $3'$). The active form of NF- κ B contained in cell extracts specifically binds to this oligonucleotide and can be detected by an ELISA technique. The ELISA kit contains a positive control from Jurkat cells stimulated with TPA and calcium ionophore.

Histological analysis

Full-thickness sections of $5\text{ }\mu\text{m}$ were stained with hematoxylin and eosin and graded by three pathologists (Ana Nieto, Angel Concha and Maria Dolores Lorente) blinded to the experimental groups according to the criteria described previously by Stucchi *et al.* (2000) (Table 2). In the colonic samples obtained from the treatment protocol with quercitrin on established colitis, immunolabelling was also performed on formalin-fixed paraffin wax-embedded samples using the streptavidin-biotin peroxidase complex method and a high-temperature pretreatment as antigen-unmasking protocols. After pretreatment, sections were incubated 15 min with 1% hydrogen peroxidase to block endogenous peroxidase activity, and were preincubated with protein-blocking sera for 30 min to reduce nonspecific reactions. The primary antibody used was a rabbit

Table 2 Scoring criteria of full-thickness distal colon sections

Mucosal epithelium
Ulceration: none (0); mild surface (1); moderate (2); extensive-full thickness (3)
Crypts
Mitotic activity: lower third (0); mild mid-third (1); moderate mid-third (2); upper third (3)
Neutrophilic infiltrate
Mucus depletion
Lamina propria
Plasmacytoid infiltrate
Neutrophilic infiltrate
Vascularity
Fibrin deposition: none (0); mucosal (1); submucosal (2); transmural (3)
Submucosal
Neutrophilic infiltrate
Edema
Scoring scale: 0, none; 1, mild; 2, moderate; 3, severe.
Maximum score: 30. Modified from Stucchi <i>et al.</i> (2000).

polyclonal anti-iNOS (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.; dilution of 1:100, incubated overnight at 4°C). The revealed system (Dako immunoreactivity) was visualized with 3-3' diaminobenzidine tetrachloride (Sigma Chemical Co., St Louis, U.S.A.) and hydrogen peroxide (0.01%), and slides were counterstained for 2 min with Gill's hematoxylin. Dilutions and washing were made in Tris-buffered saline (TBS). Negative control slides were made by substituting the primary antibody with TBS or with normal swine serum. The indirect immunofluorescence methods were performed according to established procedures. The primary mouse monoclonal antibodies used were anti-rat mononuclear phagocytes (BD Pharmingen, clone 1C7), anti-rat granulocytes (BD Pharmingen, clone RP-1) and anti-rat CD3 (BD Pharmingen, clone G4.18). Primary antibodies were diluted 1:40 in TBS and incubated in a humidity chamber overnight at 4°C . Samples were later incubated with appropriate FITC-conjugated secondary antibodies (Dako Co. Inc.) diluted 1:30 at 37°C . Sections were then mounted with Aquatex® (Merck and Co. Inc.).

Protein extraction and Western blot analysis

The colonic samples obtained from rats were homogenized ($1/3\text{ w v}^{-1}$) in PBS supplemented with 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium deoxycholate, 1% Triton X-100 and protease and phosphatase inhibitors. The proteins ($100\text{ }\mu\text{g}$) were boiled at 95°C in Laemmli SDS-loading buffer and separated on 7.5% SDS-PAGE for the detection of iNOS or eNOS. The proteins were then electrotransferred to nitrocellulose membranes (PROTAN®, Schleicher & Schuell, Germany). The membranes were blocked for at least 1 h at room temperature in TBS-0.1% Tween-20 (TBS-T) 5% nonfat dry milk and then incubated with TBS-T containing BSA 5% and the following dilution of each primary antibody: 1:2000 iNOS (Transduction Laboratories, Becton Dickinson Biosciences, Madrid, Spain) and 1:2000 eNOS (Santa Cruz Biotechnology,

Santa Cruz, CA, U.S.A.) overnight at 4°C. After three washes of 5 min each with TBS-T, the membranes were incubated with 5% nonfat dry milk and peroxidase-conjugated anti-rabbit IgG antibody for 1 h (1:5000). After three washes of 5 min with TBS-T, ECL detection was performed (NEN Life science Products, Zaventem, Belgium). Control of protein loading and transfer was conducted by detection of the β -actin levels.

Drugs and reagents

Quercitrin was purchased from Extrasynthèse (Genay, France). DSS was provided by ICN Biomedicals (Costa Mesa, CA, U.S.A.), glutathione reductase by Böehringer Mannheim (Barcelona, Spain) and *p*-nitrophenylphosphate by Merck (Madrid, Spain). All other reagents, unless otherwise stated, were obtained from Sigma (Madrid, Spain).

Statistical analysis

All results are expressed as mean \pm s.e.m. Differences among means were tested for statistical significance using one-way analysis of variance (ANOVA) and *post hoc* least significance tests. Statistical significance was set at $P < 0.05$.

Results

Preventative effect of quercitrin in DSS colitis

Exposure to DSS in the drinking water at a concentration of 5% ($w v^{-1}$) for 7 days induced a colonic inflammatory status, with features similar to those described previously (Stucchi *et al.*, 2000; Kullmann *et al.*, 2001). Half of the animals developed loose stools from the first day proceeding from the start of the DSS administration, which turned to diarrhea in the vast majority of them (90–100%) after 4–5 days. By 6–7 days, gross rectal bleeding was evident in 70% of the colitic control group rats. The inflammatory process was also assessed by a progressive loss of weight in all animals associated with anorexia, evident from the first day after the beginning of DSS administration, and that progressively increased during the course of the experiment. Thus, the DAI was increased in control colitic rats throughout the experiment (Figure 1). There were no significant differences in water consumption among all the groups over the period of the experiment, the average volume of daily water consumption being 20.5 ± 0.7 ml per rat for all groups.

Macroscopic examination of the colonic specimens after 7 days of DSS treatment revealed bowel wall thickening with a

significant increase of the colonic weight/length ratio in comparison with noncolitic rats (112.1 ± 7.1 vs 76.2 ± 1.9 mg cm^{-1} ; $P < 0.01$). The histological examination of colonic sections also assessed the intestinal inflammatory status, which showed severe inflammatory changes in the mucosa, including ulceration and loss of normal crypt architecture with necrosis and crypt abscesses. Goblet cells appeared depleted from their mucin content and inflammatory cell infiltration was prominent in all the layers of the intestine. In addition, submucosal edema and vascular hyperplasia were observed in all the samples, whereas fibrin deposition, together with residual proteins, was noted on the necrosed epithelium in 50% of the rats. According to the criteria described in Table 2, control colitic rats showed a tissue damage score of 19.4 ± 1.4 . The biochemical determinations are shown in Table 3, and revealed that DSS administration enhanced colonic MPO and AP activities, increased colonic LTB_4 levels, as well as promoted glutathione depletion.

Oral treatment of colitic rats with quercitrin (1 or 5 mg kg^{-1}), when starting the same day as DSS administration, ameliorated the inflammatory process, although it was more patent in the group of colitic rats treated with the lowest dose of flavonoid. During the course of the experiment, colitic animals treated with quercitrin at the dose of 1 mg kg^{-1} had a significantly attenuated response compared to the nontreated control group, showing a significant decrease in DAI values

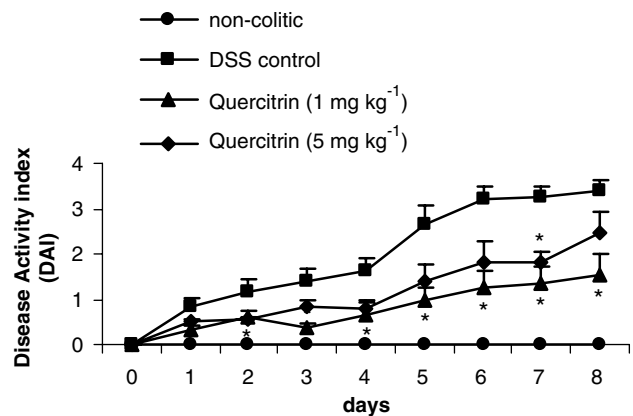


Figure 1 Preventative effects of quercitrin treatment (1 or 5 mg $kg^{-1} day^{-1}$) on the time-course changes in the DAI over the 8-day experimental period of DSS model of rat colitis, based on the criteria shown in Table 1. Treatment with quercitrin (5 mg $kg^{-1} day^{-1}$) significantly attenuated the evolution of the colonic inflammatory process from day 2 up to the end of the study. * $P < 0.05$ vs DSS control group.

Table 3 Preventative effects of quercitrin treatment (1 or 5 mg kg^{-1}) on myeloperoxidase (MPO) activity, alkaline phosphatase (AP) activity, glutathione (GSH) content and LTB_4 synthesis in the DSS model of rat colitis

Group	MPO (U g^{-1} tissue)	AP (mU mg^{-1} protein)	GSH (nmol g^{-1} tissue)	LTB_4 (ng g^{-1} tissue)
Noncolitic	5.6 ± 0.3	6.7 ± 0.3	1665 ± 47	2.4 ± 0.3
DSS control	158.8 ± 22.0	15.1 ± 1.6	1028 ± 66	5.5 ± 0.4
DSS quercitrin				
1 mg kg^{-1}	$61.0 \pm 5.2^{**}$	$10.5 \pm 1.6^{**}$	$1310 \pm 55^{**}$	4.9 ± 0.4
5 mg kg^{-1}	119.8 ± 10.2	14.1 ± 1.8	$1416 \pm 73^{**}$	4.8 ± 0.6

Data are expressed as mean \pm s.e.m. ** $P < 0.01$ vs DSS control group. All groups differ significantly from the noncolitic group ($P < 0.01$, not shown), except for glutathione levels in quercitrin-treated groups.

($P < 0.05$; Figure 1), which was evident from day 2 to 8, whereas at the dose of 5 mg kg^{-1} there was only a significant reduction at day 7. Once the rats were killed, macroscopic evaluation of the colonic segments showed that treatment of colitic rats with quercitrin significantly reduced the weight/length ratio in comparison with nontreated colitic animals ($84.9 \pm 7.1 \text{ mg cm}^{-1}$ at 1 mg kg^{-1} and $92.6 \pm 6.9 \text{ mg cm}^{-1}$ at 5 mg kg^{-1} vs $112.1 \pm 7.1 \text{ mg cm}^{-1}$ in control group; $P < 0.05$). Histological examination of the colonic specimens confirmed the intestinal anti-inflammatory effect exerted by the flavonoid. Thus, most of the animals (5/8) from the group of rats that received the lowest dose of quercitrin (1 mg kg^{-1}) showed a reduction in the areas of ulcerated colonic mucosa, affecting only 25% of the surface, without presenting fibrin deposition in the necrosed areas of the colon. The crypts showed a nearly normal architecture and goblet cell component compared with nontreated colitic rats. Although the presence of granulocytes was observed in the lamina propria, this was considered as slight or moderate in the majority of the sections evaluated, whereas macrophages and plasmatic cells were almost absent in lamina propria and submucosa. The administration of quercitrin to DSS colitic animals reduced the tissue damage score by approximately 35% compared to nontreated colitic rats (12.6 ± 2.5 vs 19.4 ± 1.4 ; $P < 0.05$). On the contrary, the histological analysis of the samples from colitic animals treated with 5 mg kg^{-1} of quercitrin revealed that the amelioration in crypt architecture was the unique parameter improved, without showing statistical differences in the damage score values in comparison with nontreated colitic rats (18.3 ± 1.8 vs 19.4 ± 1.4 ; $P > 0.1$).

The preventative intestinal anti-inflammatory effect showed by quercitrin was also assessed biochemically. In comparison with nontreated colitic animals, significant inhibition of both AP and MPO activities by 54 and 57%, respectively, were observed after flavonoid treatment at the dose of 1 mg kg^{-1} (Table 3). The latter suggests the reduction in the infiltration of neutrophils into the colonic mucosa, since MPO activity is considered as a biochemical marker of neutrophil infiltration (Krawisz *et al.*, 1984). In addition, quercitrin treatment in colitic rats partially counteracted the colonic glutathione depletion (Table 3), thus preserving the colonic tissue from the oxidative damage that characterizes intestinal inflammation. However, quercitrin treatment failed to significantly inhibit colonic LTB_4 production (Table 3) in the colonic inflamed tissue at any of the doses assayed.

Effects of quercitrin treatment on DSS colitis

To examine whether the beneficial effect showed by quercitrin might be ascribed to a chemical interaction with DSS rather than to a real anti-inflammatory effect, we tested the effects of quercitrin in the same model of DSS rat colitis, but in a different dosing protocol. For this purpose, rats were given 5% (w/v) DSS dissolved in the drinking water for 5 days, and then 2% (w/v) for the following 10 days. Quercitrin, at the dose of 1 mg kg^{-1} which was shown the most effective in the preventative dosing protocol, was given orally to colitic rats beginning on day 6 to evaluate the effects of the flavonoid on the ongoing colitis.

Once the rats from the DSS control group were killed, histological examination of the colonic samples revealed that the inflammatory process showed characteristics similar to

those described previously after 7 days of 5% DSS treatment. Thus, bowel wall thickening was evident macroscopically with a significant increase in the colonic weight/length ratio in comparison with noncolitic rats (101.2 ± 5.6 vs $74.5 \pm 2.1 \text{ mg cm}^{-1}$; $P < 0.01$). Microscopically, the samples from nontreated colitic rats showed typical inflammatory changes in the colonic architecture: ulceration, crypt dilation, goblet cell depletion as well as mixed cell infiltration, mainly composed by mononuclear cells (macrophages, lymphocytes and plasmatic cells), although granulocytes were also present (Figure 2). According to the criteria in Table 2, the tissue damage score assigned to these rats was 12.4 ± 2.8 . As expected, all biochemical parameters studied were significantly modified as a consequence of the inflammatory process, revealing increased colonic MPO and AP activities, higher colonic LTB_4 levels and depletion of glutathione content (Table 4).

The results obtained after daily administration of quercitrin (1 mg kg^{-1}) in this experimental protocol of chronic colitis revealed that this compound effectively exerted an intestinal anti-inflammatory effect. This was already observed, in comparison with the nontreated colitic group, during the course of the experiment, by a significant reduction in DAI values from day 7 onwards (Figure 3). The macroscopic examination of the colonic segments showed no statistical differences in the weight/length ratio between both colitic groups ($101.2 \pm 5.6 \text{ mg cm}^{-1}$ in nontreated vs $95.6 \pm 4.9 \text{ mg cm}^{-1}$ in quercitrin-treated; $P = 0.5$). However, the histological analysis revealed that quercitrin treatment improved the colonic architecture in comparison with nontreated colitic rats, and they were assigned a tissue damage score of 5.7 ± 1.9 ($P < 0.05$ vs nontreated colitic group). Thus, most of the treated rats showed an almost complete restoration of the damaged colon, displaying a moderate inflammatory infiltrate with a focal distribution, whereas only two of the rats showed signs of gross ulceration with an important inflammatory cell infiltrate. There was also a restoration in the crypt architecture with goblet cell replenishment mucin (Figure 2). Biochemically, quercitrin treatment inhibited 60 and 68% MPO and AP activities, respectively, compared to the DSS control group, and restored glutathione content (Table 4). No significant modifications were seen in LTB_4 levels after flavonoid treatment in comparison with the colitic control group (Table 4).

Effect of quercitrin on colonic NOS activity and NF- κ B activation

In the recent years, NO has been considered as an important pro-inflammatory mediator playing a key role in the pathogenesis of IBD (Salas *et al.*, 2002). For this reason, we evaluated the effect of quercitrin on colonic NOS activity in the DSS model of experimental colitis, using both treatment protocols. As expected, and according to previous studies (Obermeier *et al.*, 1999), DSS administration to rats, during either 8 or 15 days, produced an increase in colonic NOS activity (Figures 4a and 5a), as well as enhanced expression of iNOS, as detected by Western blot (Figures 4b and 5b). The inflammatory process was not associated with a significant modification in the expression of the constitutive isoform of NOS (eNOS), as evidenced by Western blot (Figure 5b). When the colonic homogenates from control colitic animals were

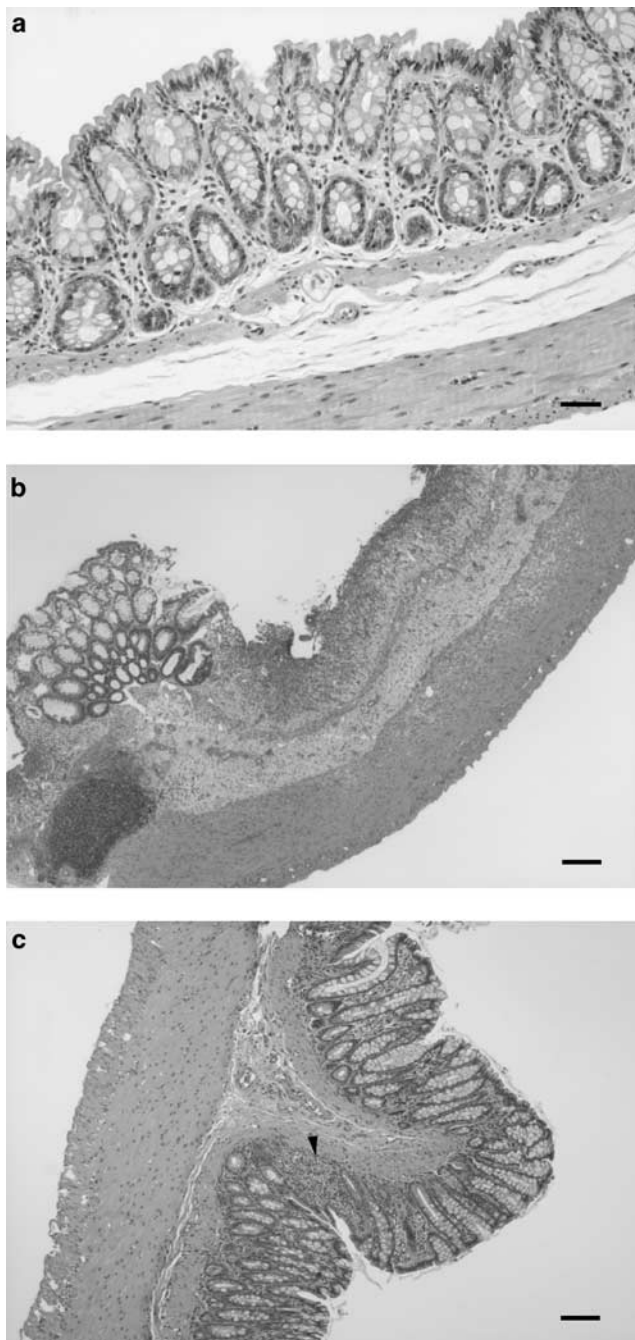


Figure 2 Histological sections of colonic mucosa from DSS colitic rats stained with hematoxylin and eosin, showing the effects of quercitrin treatment when administered after an initial colonic damage with the offending agent. (a) Noncolitic group showing the normal histology of the rat colon (bar: 60 μm). (b) DSS control group showing extensive intestinal ulceration with a severe inflammatory cell infiltrate in the lamina propria and submucosa (bar: 200 μm). (c) Quercitrin-treated group (1 mg kg^{-1}) showing recovery in the inflammatory process without ulceration of the intestinal mucosa; only a small focus of inflammatory cells can be observed (arrow) (bar: 200 μm).

incubated in the presence of the selective iNOS inhibitor aminoguanidine (10^{-4} M) (Griffiths *et al.*, 1993), a significant inhibition was observed in their enzyme activity (58.3 ± 8.9 vs 97.8 ± 6.1 pmol citrulline mg protein^{-1} ; $P < 0.05$), showing similar values to those from noncolitic rats (53.5 ± 8.9 pmol

citrulline mg protein^{-1} ; $P > 0.05$). The intestinal anti-inflammatory effect exerted by quercitrin, both in the preventative and in the curative dosing protocols, was associated with a significant inhibition of colonic NOS activity (Figures 4a and 5a), and a reduction of iNOS expression (Figures 4b and 5b) when compared to DSS control animals.

According to the well-described role of NF- κ B in inflammatory regulation (Schottelius & Baldwin, 1999) and in iNOS expression (Xie *et al.*, 1994), the degree of NF- κ B activation was determined in the colonic tissue samples from animals submitted to DSS administration for 15 days. Actually, the inflammatory status induced by DSS was associated with a significant increase of colonic NF- κ B activity when compared to noninflamed tissue (Figure 5c). NF- κ B activity was down-regulated to normal values after quercitrin treatment (Figure 5c).

Involvement of activated macrophages in colonic inflammation

In normal intestinal mucosa from noncolitic rats, we observed minimal immunofluorescence for macrophages (Figure 6a), neutrophils and T lymphocytes (not shown), and a weak immunostaining to iNOS (Figure 7a).

However, immunofluorescence staining of colonic samples from control group of rats that received DSS for 15 days revealed that the predominant cells in the inflammatory areas of the intestine were macrophages, which were mainly located in the lamina propria and the submucosa-adjointing regions of ulceration (Figure 6b). In addition to these cells, neutrophils were also found in crypt abscesses and in the surface of ulcerated epithelium, although in a lesser extent than macrophages (not shown). The presence of T cells in the inflammatory infiltrate was rare (not shown). Moreover, immunostaining to iNOS had a similar distribution in the colonic samples from the nontreated colitic group. Of note, all macrophages, the polymorphonuclear leukocytes infiltrating the lamina propria around the ulcers and the lymphocytes located at the base of the ulcers showed an activated phenotype since immunohistochemistry against iNOS was positive in these cells (Figure 7b). Immunostaining of iNOS was also located at the bases of the epithelial crypts in brush border of the cytoplasm (Figure 7b).

The intestinal anti-inflammatory effect exerted by quercitrin was associated to a decreased number of infiltrated macrophages (Figure 6c) and neutrophils (not shown) in comparison with nontreated colitic rats, as evidenced by immunofluorescence staining of the colonic samples. This was also observed by hematoxylin and eosin staining (Figure 2). T-cell contribution to the inflammatory infiltrate was not modified after quercitrin treatment (not shown). Moreover, the epithelial and inflammatory cells showed a weaker intensity of immunostaining to iNOS (Figure 7c) in quercitrin-treated rats than in the corresponding colitic rats without treatment, and more similar to that found in normal intestinal mucosa.

Discussion

The present study describes that oral administration of quercitrin, at the dose of 1 mg kg^{-1} , significantly prevented the progression of DSS-induced colitis in rats when the

Table 4 Effects of quercitrin treatment on established colitis (1 mg kg^{-1}) on myeloperoxidase (MPO) activity, alkaline phosphatase (AP) activity, glutathione (GSH) content and LTB_4 synthesis in the DSS model of rat colitis

Group	MPO (U g^{-1} tissue)	AP (mU mg^{-1} protein)	GSH (nmol g^{-1} tissue)	LTB_4 (ng g^{-1} tissue)
Noncolitic	7.8 ± 0.3	7.5 ± 0.3	1787 ± 35	2.2 ± 0.2
DSS control	$127.2 \pm 14.7^{++}$	$14.5 \pm 1.4^{++}$	$1517 \pm 49^{++}$	$7.3 \pm 0.8^{++}$
DSS quercitrin	$58.5 \pm 8.9^{++,*}$	$9.8 \pm 0.5^{++,*}$	$1762 \pm 67^{**}$	$5.8 \pm 0.3^+$

Data are expressed as mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$ vs DSS control group. + $P < 0.05$, ++ $P < 0.01$ vs noncolitic group.

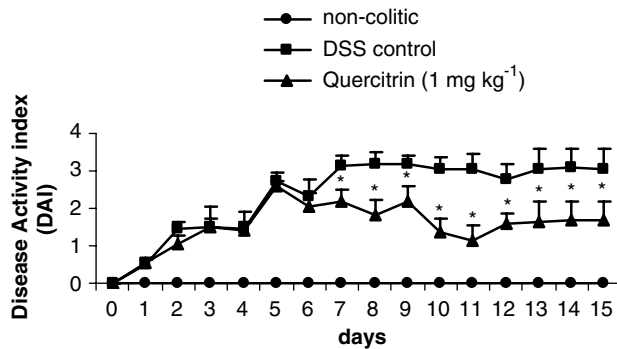


Figure 3 Effects of quercitrin treatment ($1 \text{ mg kg}^{-1} \text{ day}^{-1}$) on the time-course changes in the DAI over the 15-day experimental period in the DSS model of rat colitis, based on the criteria shown in Table 1. Treatment with quercitrin significantly attenuated the evolution of the colonic inflammatory process from day 7 up to the end of the study. * $P < 0.05$; ** $P < 0.01$ vs DSS control group.

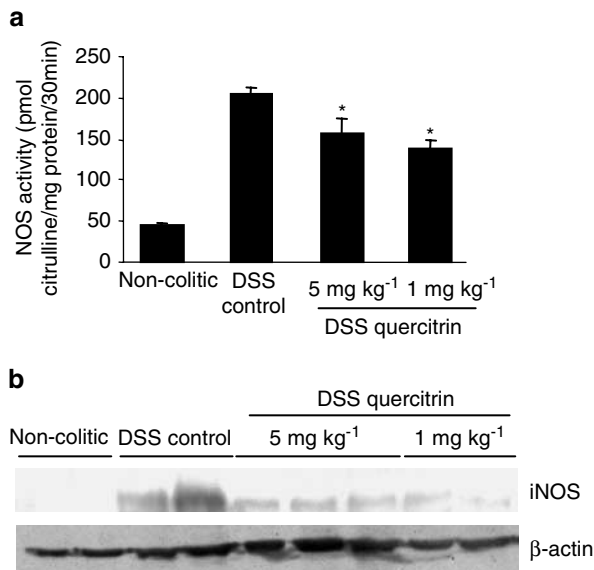


Figure 4 (a) Effect of quercitrin treatment (1 or 5 mg kg^{-1}) on colonic NOS activity in DSS colitic rats in preventative treatment; * $P < 0.05$ vs DSS control rats. All colitic groups differ significantly from the noncolitic group ($P < 0.01$, not shown). (b) Measurement of iNOS expression by Western blotting of colonic protein samples from noncolitic rats, DSS control rats and DSS quercitrin-treated rats (1 or 5 mg kg^{-1}) in the preventative dosing protocol.

compound was administered from the day DSS was added to the drinking water. However, this dosing protocol did not allow us to distinguish between a real anti-inflammatory effect and any possible artefact due to a physical impediment in the

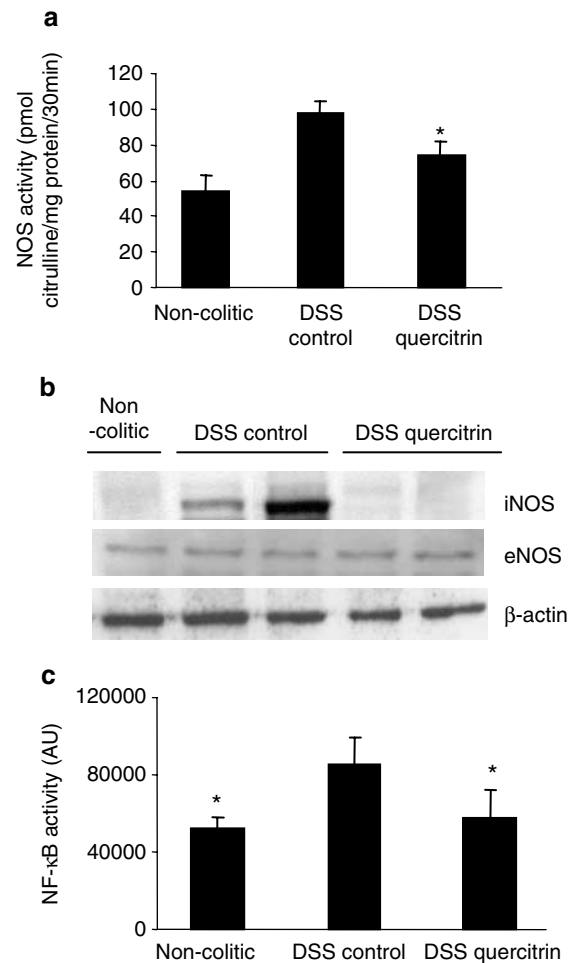


Figure 5 Effects of quercitrin treatment ($1 \text{ mg kg}^{-1} \text{ day}^{-1}$) on established colitis: (a) Colonic NOS activity in DSS colitic rats; * $P < 0.05$ vs DSS control rats; all colitic groups differ significantly from the noncolitic group ($P < 0.01$, not shown). (b) Measurement of iNOS and eNOS expression by Western blotting in DSS colitic rats. (c) NF- κ B activity in DSS colitic rats; * $P < 0.05$ vs DSS control rats (Kruskal–Wallis one-way ANOVA, followed by Student–Newman–Keuls test).

damage effect of DSS, or to a delay in the appearance of the colonic damage. These facts led us to undertake additional experiments, in which we assayed the same active dose of quercitrin following a different dosing protocol in which the administration of the flavonoid was started after 5 days of 5% DSS administration in the drinking water when the colonic damage had been established. The results obtained with these assays effectively revealed that quercitrin treatment, during 10 days, facilitated the recovery of the inflammatory process induced by DSS, even when the administration of DSS was

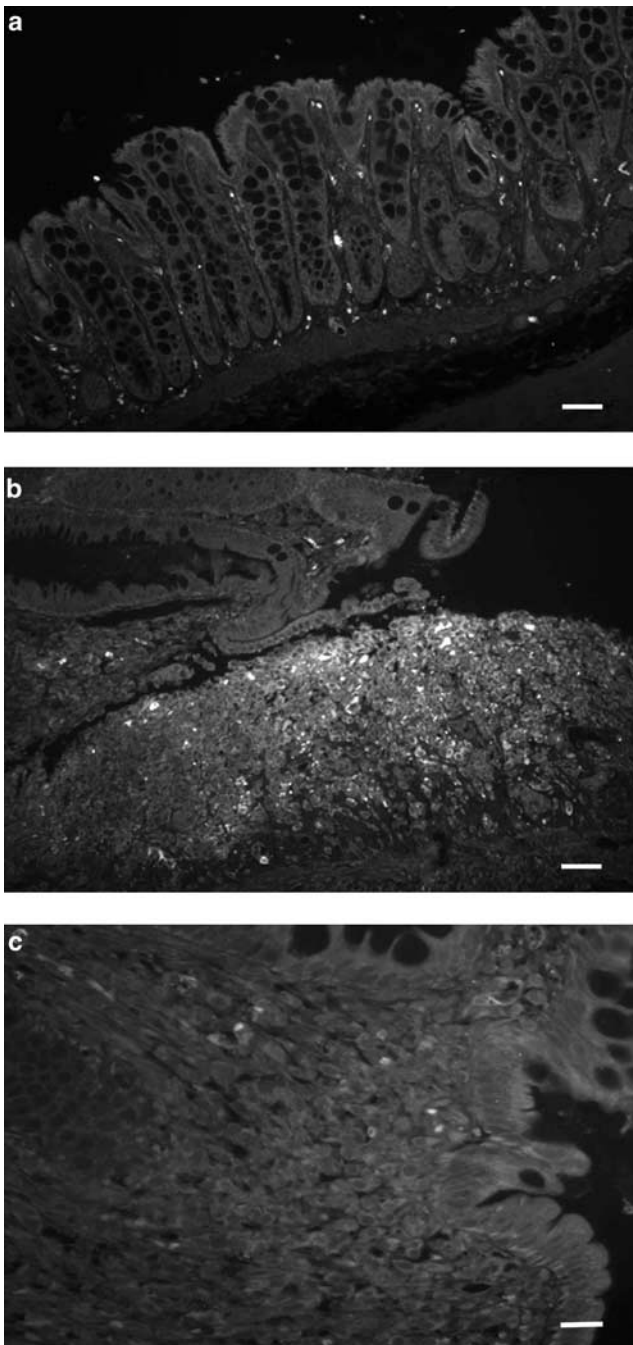


Figure 6 Immunofluorescence of macrophages with clone 1C7 performed in histological sections of colonic mucosa from DSS colitic rats in the treatment protocol with quercitrin on established colitis. (a) Noncolitic group showing scarce expression in the colonic tissue (bar: 60 μm). (b) DSS control group showing greatly increased expression in an ulcerated area (bar: 60 μm). (c) Quercitrin-treated group (1 mg kg⁻¹) showing the decreased in the immunoreactivity to macrophages (bar: 30 μm).

maintained to all colitic animals, but at a lower concentration (2%). The beneficial effects observed in both dosing protocols were assessed both clinically, by improvement of weight loss and bloody diarrhea, and histologically by preservation of the intestinal architecture in comparison with the rats from the nontreated colitic group. The biochemical assays performed in the colonic specimens confirmed the anti-inflammatory effect

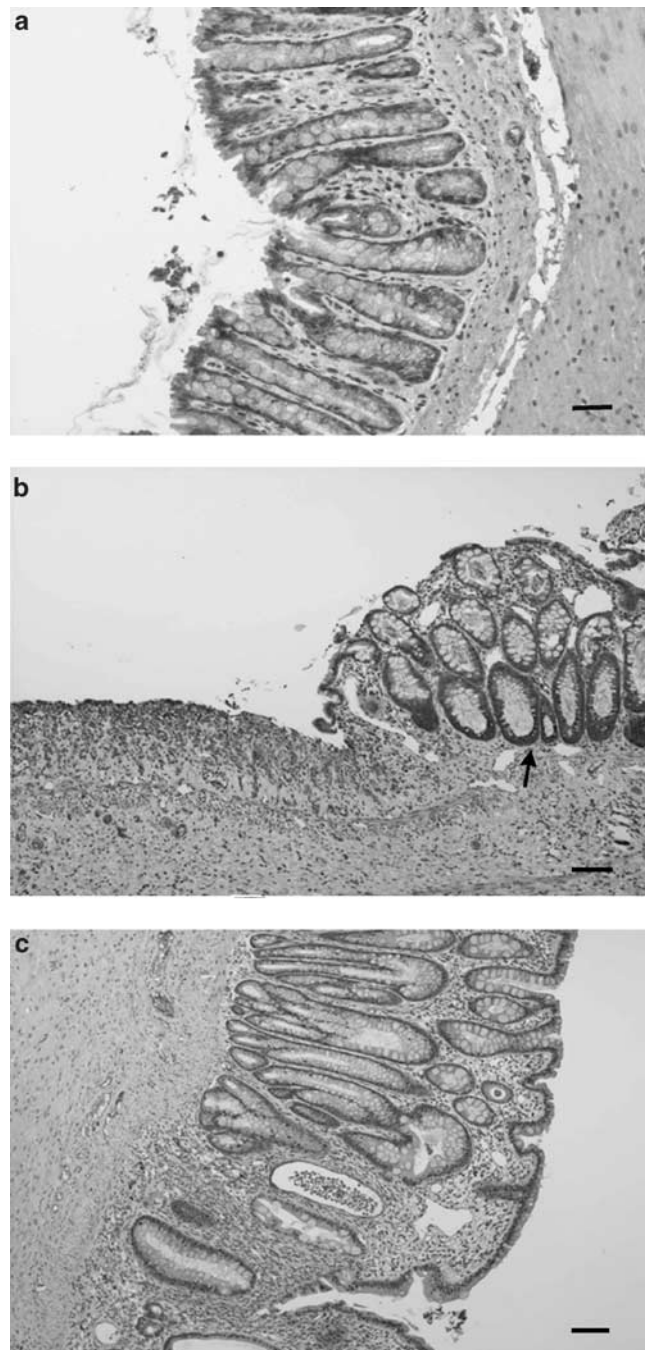


Figure 7 Immunostaining of iNOS performed in histological sections of colonic mucosa from DSS colitic rats in the treatment protocol with quercitrin on established colitis (bar: 200 μm). (a) Noncolitic group; (b) DSS control group showing increased immunoreactivity to iNOS; (c) Quercitrin-treated group (1 mg kg⁻¹) showing the decreased in the immunoreactivity to iNOS.

exerted by quercitrin at the dose of 1 mg kg⁻¹, since it was associated to a significant reduction in MPO and AP activities. MPO activity has been widely used to detect and follow intestinal inflammation, and a reduction in the activity of this enzyme can be interpreted as a manifestation of the anti-inflammatory activity of a given compound (Veljaca *et al.*, 1995). Similarly, and based on our previous studies performed in two different models of experimental colitis, AP activity can also be considered as a sensitive marker of inflammation in the

intestine, since this enzyme activity is invariably augmented in these experimental conditions (Sanchez de Medina *et al.*, 1996; Galvez *et al.*, 1997). The results obtained in the present study confirm the intestinal anti-inflammatory effect previously demonstrated for this flavonoid in the TNBS model of rat colitis (Sanchez de Medina *et al.*, 1996; 2002), which more closely resembles Crohn's disease (Fuss *et al.*, 1999). On the contrary, the rat model of DSS-induced colitis is a well-characterized model with a predictable disease progression, which shares numerous clinical, biochemical and histological features with human ulcerative colitis (Gaudio *et al.*, 1999; Kullmann *et al.*, 2001). In consequence, the fact that quercitrin has shown beneficial effects in two different models of rat colitis makes this flavonoid a good candidate to be developed in human IBD therapy, even more if we consider the low toxicity associated with the consumption of this type of product, which is present in the normal diet in significant amounts (Middleton *et al.*, 2000).

In addition, the present study gives some clues about the mechanisms involved in the intestinal anti-inflammatory effect of quercitrin. One of these mechanisms could be the inhibition of free radical generation exerted by quercitrin, given the well known antioxidant properties ascribed to this flavonoid, as well as to its aglycone quercetin, both *in vitro* and *in vivo* (Galvez *et al.*, 1994; Bors *et al.*, 1990). This activity may play a crucial role in the intestinal anti-inflammatory effect of the flavonoid, because a situation of intense oxidative insult is a common feature in human IBD (Grisham, 1994; McKenzie *et al.*, 1996) and in the different experimental models of rat colitis, such as the TNBS (Sanchez de Medina *et al.*, 1996; Galvez *et al.*, 2001) and the DSS (Stucchi *et al.*, 2000) models, and is an important mechanism for tissue damage during chronic intestinal inflammation. In fact, previous studies have shown that the antioxidant and/or radical scavenging properties of quercitrin may contribute to its beneficial effects when assayed in the initial phase of the TNBS-induced colonic damage (Sanchez de Medina *et al.*, 1996; 2002). In this regard, in the present study, quercitrin treatment of DSS colitic rats counteracted the depletion of colonic glutathione levels that took place in control colitic animals in both experimental treatment protocols. The effect exerted by quercitrin in preserving the colonic mucosa from oxidative insult may collaborate to decrease the neutrophil infiltration that occurs in response to DSS. Free radical generation has been proposed to play an important role early on in the pathogenesis of IBD (Pavlick *et al.*, 2002), and could contribute to the initial neutrophil infiltration in the inflamed colonic mucosa. The recruitment and activation of these cells results in a dramatic increase in free radical production that overwhelms the tissular antioxidant protective mechanisms, resulting in a situation of oxidative stress, which definitively perpetuates colonic inflammation (Grisham, 1994). As a consequence, a rapid inhibition of free radical generation could contribute to a lower level of leukocyte infiltration into the inflamed tissue, thus preventing colonic tissue from inflammation. It is important to note that quercitrin could attenuate neutrophil infiltration *via* inhibition of other different mediators with chemotactic activity, given the reported ability of this type of natural products to modulate the immune response through downregulation of different pro-inflammatory mediators such as eicosanoids (Middleton *et al.*, 2000). However, no inhibitory effect was observed on colonic LTB₄ production, an eicosanoid with

chemotactic properties, whose production has been reported to be increased in these intestinal conditions (Sharon & Stenson, 1984). These results are in agreement with previous studies in which no correlation was observed between the ability of quercitrin to downregulate colonic LTB₄ production and its anti-inflammatory effect in the acute phase of the TNBS model of rat colitis (Sanchez de Medina *et al.*, 1996).

During the last decade, it has become increasingly clear that NO overproduction by iNOS is deleterious to intestinal function (Hogaboam *et al.*, 1995; Rachmilewitz *et al.*, 1995; Kimura *et al.*, 1997; Salas *et al.*, 2002), thus contributing significantly to gastrointestinal immunopathology during the chronic inflammatory events that take place in IBD (Grisham *et al.*, 2002). The important role attributed to NO in these intestinal conditions prompted us to study whether the beneficial effects of quercitrin on DSS-induced colitis could be related to an effect on colonic NO production. As expected, the results obtained in the present study reveal that colonic inflammation is associated with a higher colonic NOS activity, mainly attributed to an increase in iNOS expression, as evidenced by Western blot analysis. Two experimental findings support this fact: first, when additional experiments were performed in which the colonic homogenates from colitic animals were incubated in the presence of the selective iNOS inhibitor aminoguanidine (10⁻⁴ M) (Griffiths *et al.*, 1993), a reduction in NOS activity was obtained, showing similar activity to that obtained in specimens from noncolitic rats; second, colonic eNOS expression was not significantly modified by the inflammatory insult induced with DSS, as evidenced by Western blot analysis (Figure 5b). The increase in iNOS expression in colitic animals in comparison with noncolitic samples was confirmed by the immunohistochemistry analysis (Figure 6). This probably results from the intense activation of macrophages, which took place as a consequence of the inflammatory insult, as also evidenced by the immunofluorescence studies in the intestinal specimens from colitic animals. In fact, macrophages are considered an important source of pro-inflammatory mediators, such as NO and TNF α , playing a key role in the pathophysiology of IBD (Neurath *et al.*, 1996).

Quercitrin treatment to colitic rats effectively inhibited the upregulated colonic NOS activity, being able to reduce iNOS expression as evidenced both by Western blotting and by immunohistochemistry. The inhibition of NOS activity could also be ascribed to a direct inhibition in this enzyme activity based on the ability of flavonoids to inhibit different enzyme activities, including NOS (Middleton *et al.*, 2000). However, when homogenates from colonic inflamed tissue from colitic rats that received DSS (5% p v^{-1}) for 5 days (as previously described) were used as a NOS activity source, quercitrin or its aglycone quercetin (which is released from quercitrin *in vivo*) showed a weak inhibitory activity (unpublished results). These results are in agreement with previous studies performed by Kim *et al.* (1999), who demonstrated a lack of direct inhibitory effect of quercetin or its glycosides on iNOS activity in the macrophage cell line RAW 264.7. In consequence, and in concordance with the results obtained in the present study, the effect showed by quercitrin is most probably related to an inhibition of the expression of this enzyme, which is upregulated as a consequence of the colonic inflammatory process. In fact, the inhibition of iNOS expression by flavonoids has been previously reported in the macrophage

cell line RAW 264.7, having been suggested that this occurs by preventing the activation/translocation of NF- κ B (Kim *et al.*, 1999; Tsai *et al.*, 1999).

NF- κ B, which plays a key role in the activation of genes involved in immune responses (Baldwin, 1996), is an attractive candidate for being involved in IBD pathogenesis. The results obtained in the present study revealed that oral treatment of quercitrin to colitic rats resulted in a significant inhibition of the NF- κ B pathway, which was activated as a consequence of the intestinal inflammatory process induced by DSS. The molecular mechanisms involved in the suppressive effects of flavonoids on NF- κ B are currently unknown, but several possibilities have been postulated. The first possibility is that flavonoids may inhibit NF- κ B by acting as antioxidants, since NF- κ B is a redox-sensitive transcription factor and activated by oxidant stress in the inflamed intestinal mucosa (Rogler *et al.*, 1998). The second possibility is that flavonoids inhibit NF- κ B *via* blocking the phosphorylation as well as degradation of I κ B protein, a crucial step in the translocation of NF- κ B to the nucleus and its subsequent activation, as it has been previously reported *in vitro* (Tsai *et al.*, 1999).

Finally, it is important to note that increasing doses of the flavonoid resulted in a decreased intestinal anti-inflammatory effect as it has been observed in the preventative dosing protocol (5 mg kg⁻¹), similar to what has been previously described for this compound (Sanchez de Medina *et al.*, 1996), as well as for other flavonoids like hesperidin and diosmin (Crespo *et al.*, 1999) in the TNBS model of rat colitis. The loss of activity may be related to the reported capacity of flavonoids, including quercetin, to inhibit cyclooxygenase when the doses are increased (Kim *et al.*, 1998), an effect described for nonsteroidal anti-inflammatory drugs, which

that way can induce an exacerbation of the inflammatory process in experimental colitis (Reuter *et al.*, 1996). We have also to consider the capacity of quercetin to autooxidize *in vitro* under some circumstances (Canada *et al.*, 1990; Lopez-Lopez *et al.*, 2004), and generate free radicals that could outweigh the beneficial effects of quercitrin at lower doses.

In conclusion, quercitrin treatment ameliorates colonic damage in DSS-induced colitic rats, an effect associated with an improvement in intestinal oxidative stress and a down-regulation in colonic NOS activity mediated by the reduction of iNOS protein expression. The iNOS inhibition produced by quercitrin is correlated with the inhibition of NF- κ B activity. The inhibition of these intermediates contributes to the resolution of exacerbated inflammation produced by experimental colitis. The antioxidant and/or scavenging properties ascribed to this flavonoid could also contribute to its intestinal anti-inflammatory effect, similarly to other reputed drugs used in the treatment of IBD, such as 5-aminsalicylic derivatives (Travis & Jewel, 1994), thus supporting the future application of quercitrin in the treatment of human IBD.

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