Università degli Studi di Padova

Padua Research Archive - Institutional Repository

The β3-adrenoceptor agonist SR58611A ameliorates experimental colitis in rats

Original Citation:

Availability:
This version is available at: 11577/3171948 since: 2016-01-09T14:22:23Z

Publisher:

Published version:
DOI: 10.1111/j.1365-2982.2008.01138.x

Terms of use:
Open Access

This article is made available under terms and conditions applicable to Open Access Guidelines, as described at http://www.unipd.it/download/file/fid/55401 (Italian only)

(Article begins on next page)

 doi: 10.1111/j.1365-2982.2008.01138.x

The β_3-adrenoceptor agonist SR58611A ameliorates experimental colitis in rats

V. VASINA,* E. ABU-GHARBIEH,† G. BARBARA,‡ R. DE GIORGIO,‡ R. COLUCCI,§ C. BLANDIZZI,§ N. BERNARDINI,¶ T. CROCI,** M. DEL TACCA§ & F. DE PONTI*

*Department of Pharmacology, University of Bologna, Bologna, Italy
†Department of Biopharmaceutics and Clinical Pharmacy, University of Jordan, Amman, Jordan
‡Department of Internal Medicine and Gastroenterology, University of Bologna, Bologna, Italy
§Division of Pharmacology and Chemotherapy, Department of Internal Medicine, University of Pisa, Pisa, Italy
¶Section of Histology and Medical Embryology, Department of Human Morphology and Applied Biology, University of Pisa, Pisa, Italy
**Sanofi-Midy Research Centre, Sanofi-aventis SpA, Milan, Italy

Abstract *β_3-Adrenoceptor agonists protect against experimental gastric ulcers. We investigated the effects of the β_3-adrenoceptor agonist SR58611A on 2,4-dinitrobenzene sulphonic acid-induced colitis in rats and analysed the expression of β_3-adrenoceptors in the colonic wall. SR58611A was administered orally (1–10 mg kg^{-1}) for 7 days, starting the day before induction of colitis. Colitis was assessed by macroscopic and histological scores, tissue myeloperoxidase activity, interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) levels. Reverse transcription-polymerase chain reaction and immunohistochemical analysis were used to examine the expression of β_3-adrenoceptors. SR58611A significantly reduced the severity of colitis as well as the tissue levels of TNF-α, IL-1β and IL-6. Colitis was associated with a decreased expression of β_3-adrenoceptor mRNA in the mucosal/submucosal layer of distal colon and this reduction was not affected by SR58611A. Immunohistochemical analysis revealed β_3-adrenoceptors within the muscularis externa, in myenteric neurons and nerve fibres and in the submucosa. β_3-Adrenoceptor immunoreactivity was decreased in inflamed tissues compared to controls, particularly in the myenteric plexus; this reduction was counteracted by SR58611A. Amelioration of experimental colitis by the selective β_3-adrenoceptor agonist SR58611A suggests that β_3-adrenoceptors may represent a therapeutic target in gut inflammation.*

Keywords *adrenoceptors, colitis, enteric neurons.*

Address for correspondence
Fabrizio De Ponti MD, PhD, Department of Pharmacology, Via Irnerio, 48, I-40126 Bologna BO, Italy.
Tel: +39 051 2091805; fax: +39 051 248862;
e-mail: fabrizio.deponti@unibo.it
Received: 20 July 2007
Accepted for publication: 28 March 2008

INTRODUCTION

β_3-Adrenoceptors are widely distributed in the gastrointestinal tract of several species, including humans[1] and rats:[2] in particular, they are expressed on gut vascular and non-vascular smooth muscle, where they mediate relaxation and are probably involved in the control of blood flow.[1–5] Recently, β_3-adrenoceptors have been localized on a subpopulation of cholinergic neurons of the human colon.[6]

β_3-Adrenoceptor agonists protect against indomethacin-induced antral and jejunal ulcerations and reduce early microvascular injury, probably by increasing gastric mucosal blood flow and reversing early villous shortening by relaxation of villous smooth muscle.[7] Specifically, the β_3-adrenoceptor agonist SR58611A inhibits gastric acid secretion in cats and dogs,[8,9] and several authors reported a gastroprotective effect of the β_3-adrenoceptor agonists BRL 37344, CL316243, ZD7114, CGP12177A and SR58611A in various models of gastro-duodenal ulcers in rats, with a significant reduction in ulcer index.[7,10,11]

A loss of adrenergic influence, mainly due to a downregulation in the inhibitory β_3-adrenergic regulation, was observed in rat colonic circular smooth muscle during trinitrobenzene sulfonic acid-induced experimental colitis.[12] This mechanism might contribute to diarrhoea in patients with inflammatory bowel disease. Moreover, Blandizzi *et al.*[13] found that experimental colitis in rats enhanced the inhibitory control

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

of cholinergic and noradrenergic neurotransmission, which can be modulated by an increased expression of α_{2A}-adrenoceptors within the enteric nervous system.

Our aim was to investigate the effects of the selective β_3-adrenoceptor agonist SR58611A[2,14–16] in a rat model of colitis and to analyse the expression of β_3-adrenoceptors in the colonic wall.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (180–200 g body weight; Harlan Italy, S. Pietro al Natisone, Udine, Italy) were used in this study. Animals were housed in a controlled environment and had free access to food and water throughout the study. Before starting any experimental procedure, to minimize the effects of stress *per se* on the parameters to be measured, animals were weighed and gently manipulated in the laboratory environment for 30 min everyday for at least 1 week. All experiments were carried out according to the guidelines set forth by EEC Directive 86/609 on the care and use of experimental animals. The protocol for induction of colitis was reviewed by the Institutional Committee on the care and use of experimental animals of the University of Bologna and was authorized by the Italian Ministry of Health. A persistently hunched posture and laboured respiration, a markedly erected coat and a weight loss of more than 20% were considered as end-points to euthanize the animals.

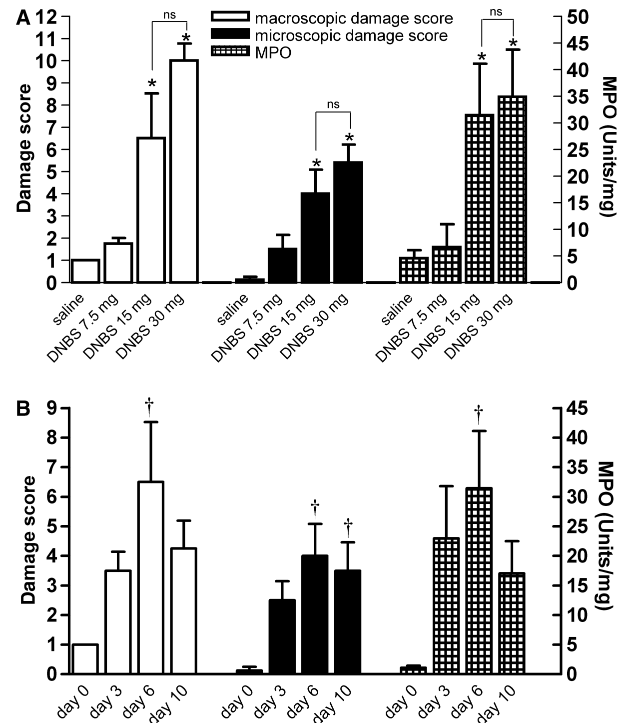

Figure 1 (A) Dose-dependent effect of DNBS (7.5, 15 and 30 mg per rat) on macroscopic damage score, microscopic damage score and MPO activity; (B) time-dependent effect of 15 mg DNBS (day 3, 6 and 10) on macroscopic damage score, microscopic damage score and MPO activity. Data are expressed as mean values ± SEM; n = 4–8 rats per group. *$P < 0.01$ *vs* saline; †$P < 0.05$ *vs* saline; ns, not significant.

Induction of experimental colitis

Colitis was induced using a previously described method[17] with slight modifications.[18] Briefly, rats were lightly anaesthetized by inhalation of chloroform (Sigma-Aldrich, Milan, Italy); 2,4-dinitrobenzene sulphonic acid (DNBS; ICN Biomedicals, 7.5, 15 or 30 mg per rat) dissolved in 0.25 mL of 50% ethanol was instilled into the distal colon of each animal using a rubber catheter, so that the tip was about 8 cm proximal to the anus. Ethanol was used as an enhancer of DNBS-induced damage but, *per se*, had no effect on the parameters to be measured.[13] Control rats received 0.25 mL 0.9% NaCl alone intrarectally. 2,4-Dinitrobenzene sulphonic acid and control rats were kept in separated cages during the study.

Experimental design

Firstly, we studied the dose–response of DNBS (7.5, 15 and 30 mg per rat) in inducing colitis. In all subsequent experiments, the dose of 15 mg of DNBS per rat was used because it evoked adequate inflammation without causing unnecessary distress and suffering to the animals (see Results and Fig. 1A).

Secondly, time-course experiments were carried out killing the animals at day 3, 6 and 10 after induction of colitis. On the basis of the results (see below and Fig. 1B), day 6, which corresponds to the time of maximal inflammatory injury,[18] was selected to kill the animals in all remaining experiments.

Thirdly, we studied groups of rats with and without colitis, which were treated with the selective β_3-adrenoceptor agonist SR58611A,[2,14–16] starting the day before the induction of colitis. SR58611A was dissolved in 0.1 N HCl solution and administered orally, by gavage, once daily at the same time. On the basis of previous studies,[2,14,16,19] in which SR58611A was shown to inhibit colonic motility at doses without effects on the cardiovascular system, we tested the following doses: 1, 3 and 10 mg kg^{-1}.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

Tissue collection

Rats were killed at different times after induction of colitis as indicated above. The distal colon was removed, opened longitudinally along the mesentery and washed with phosphate-buffered saline (PBS) to remove luminal contents. Whole-wall samples from distal-colon, taken from a region immediately adjacent to the gross macroscopic damage, were pinned flat on wax, fixed in cold neutral 4% formalin and then stored in 0.1% sodium azide at 4 °C before processing for immunohistochemistry as whole mounts (WM) or placed in 25% sucrose in PBS at 4 °C for cryoprotection and embedded in paraffin or Optimal Cutting Temperature tissue freezing medium. Five-micron-thick sections of colon were cut, serially mounted on glasses and processed for routine haematoxylin–eosin (H&E) staining or immunohistochemistry. Specimens of colonic tissue were also removed from the area of gross injury, snap frozen in liquid nitrogen and stored at −80 °C until subsequent assays. For the purpose of reverse transcription–polymerase chain reaction (RT-PCR) assays, additional tissue samples were collected from proximal uninflamed colon and stored at −80 °C.

Assessment of colonic damage

Colonic damage was assessed macroscopically and histologically using a method previously described.[20] Briefly, the macroscopic criteria were based on the following: presence of adhesions between the colon and other intra-abdominal organs, consistency of colonic faecal material (as an indirect marker of diarrhoea), thickening of the colonic wall, presence and extension of hyperaemia and macroscopic mucosal damage (expressed in mm^2). Microscopic criteria for damage and inflammation were assessed by light microscopy on H&E stained histological sections. Histological criteria included: degree of mucosal architecture changes, cellular infiltration, external muscle thickening, presence of crypt abscess and goblet cell depletion.

Assessment of myeloperoxidase activity

Myeloperoxidase (MPO) was assayed within 7 days from collection of colonic specimens using a previously described method.[21] Myeloperoxidase is a granule-associated enzyme present in neutrophils and other cells of myeloid origin, and widely used as a marker of intestinal inflammation. Colonic tissue was weighed and placed in a plastic tube with hexadecyl-trimethylammonium bromide buffer (1 mL per 50 mg of tissue), homogenized and centrifuged for 10 min at 6000 ***g*** at 4 °C. Seven microlitre of the supernatant was then collected and assayed to assess MPO activity. Myeloperoxidase was expressed in units per milligram of tissue, where 1 unit corresponds to the activity required to degrade 1 μmol of hydrogen peroxide in 1 min at room temperature.

Cytokine tissue levels

Tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) concentrations were assessed in frozen colonic samples. On thawing, samples were weighed, homogenized in a solution of protease inhibitors (pepstatin, aprotinin, leupeptin, 1 μg mL^{-1}; Sigma-Aldrich) and centrifuged at 6000 ***g*** at 4 °C. A 100-μL aliquot of supernatant was then added to the enzyme immunoassay 96-wells plate in duplicate and assayed according to the manufacturer's protocols (Rat TNF-α Ultra Sensitive kit; Rat IL-6 Immunoassay kit; Rat IL-1β Immunoassay kit; Biosource International, Camarillo, CA, USA). Absorbance was read using a spectrophotometer at 450 nm. Tumour necrosis factor-α, IL-6 and IL-1β were expressed as pg mg^{-1} of tissue.

RT-PCR analysis of β_3-adrenoceptors

There is evidence that alterations in enteric neurotransmission induced by inflammatory bowel diseases may occur at both inflamed and non-inflamed sites.[13,22] Therefore, to assess the expression of the gene coding for β_3-adrenoceptors, RT-PCR assays were performed on tissue specimens from both proximal and distal colon, to verify whether changes in β_3-adrenoceptor expression could occur in the inflamed distal colon and/or in the proximal segment. Care was taken to dissect carefully the mucosal/submucosal layer from muscular layers. At the time of extraction, tissues were disrupted in mortars refrigerated on ice. Total RNA was isolated by Trizol® (Life Technologies, Carlsbad, CA, USA) and chloroform. After denaturation (3 min at 94 °C), total RNA (5 μg) served as template for single-strand cDNA synthesis in a reaction using random hexamer oligonucleotide primers (0.05 μg μL^{-1}) with 200 U of MMLV-reverse transcriptase in first strand buffer 5 × (50 mmol L^{-1} Tris–HCl pH 8.3, 75 mmol L^{-1} KCl, 3 mmol L^{-1} $MgCl_2$) containing 800 μmol L^{-1} deoxynucleotide triphosphate mixture (dNTPs) and 5 mmol L^{-1} dithiothreitol. cDNA samples were subjected to PCR in the presence of specific rat β_3-adrenoceptor oligonucleotide primers (sense: 5′-CCACCTTGAACTTCGCTACT-3′; antisense: 5′-TTG TGCCTATTGTGAGAGAT-3′; expected size 372 base

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

pairs). PCR, consisting of 2 μL of RT products, *Taq* polymerase 2.5 U, dNTPs 100 μmol L^{-1} and oligonucleotide primers 0.1 μmol L^{-1}, was carried out by a PCR-Express thermocycler (Hybaid, Ashford, Middlesex, UK) at the following conditions: 38 cycles of denaturation at 94 °C for 15 s, annealing at 52 °C for 15 s and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. The efficiency of RNA extraction, RT and PCR were evaluated by specific sets of oligonucleotide primers for the constitutively expressed rat β-actin gene (sense: 5′-TCATGA AGTGTGACGTTGACATCCGT-3′; antisense: 5′-CTT AGAAGCATTTGCGGTGCACGATG-3′; expected size 286 base pairs). PCR cycles for β-actin were 27 under the same conditions reported above. Care was taken to verify that the number of PCR cycles for each primer set was in the linear range to perform semi-quantitative analysis of PCR products. The amplified cDNA products were separated by 1.7% agarose gel electrophoresis in a Tris buffer 40 mmol L^{-1} containing 2 mmol L^{-1} ethylenediaminetetracetic acid, 20 mmol L^{-1} acetic acid (pH 8), and stained with ethidium bromide. cDNA bands were then visualized by UV light (Bio-Rad, Richmond, CA, USA) and quantified by densitometric analysis with NIH Image computer program (Scion Corporation, Frederick, MD, USA). The relative expression of β_3-adrenoceptor mRNA was normalized to that of β-actin.

Immunohistochemistry

Anti-β_3-adrenoceptor goat polyclonal antibody (1 : 50; sc-1473, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was employed to detect β_3-adrenoceptors; anti-c-kit tyrosine kinase rabbit polyclonal (1 : 20; PC34, Calbiochem, San Diego, CA, USA) antibody was used to detect interstitial cells of Cajal (ICC).[23,24] Anti-protein gene product (PGP) 9.5 mouse monoclonal antibody (1 : 100; V3231, Biomeda, Foster City, CA, USA) was used as general neuronal marker.

For β_3-adrenoceptor immunofluorescence staining, cryostat sections were washed three times with PBS and then incubated with 10% normal donkey serum in PBS containing 0.1% Triton® X-100 to block non-specific binding. Sections were incubated with anti-β_3-adrenoceptor primary antibody in a humid chamber at 4 °C overnight, rinsed with PBS, and incubated at room temperature with Alexa Fluor® 555 donkey antigoat immunoglobulin G (IgG) (1 : 1200, Molecular Probes, Eugene, OR, USA) for 2 h. Specimens were mounted with Mowiol® 4–88 reagent (Calbiochem) and examined by fluorescence microscope (ECLIPSE 90i; Nikon Instruments, Calenzano, Italy).

For immunoperoxidase staining, paraffin sections were processed as previously described.[25] Briefly, the sections were sequentially treated as follows: microwave in sodium citrate buffer for antigen retrieval; 1% hydrogen peroxide to block endogenous peroxidase; normal goat serum (1 : 20) to block non-specific binding. Sections were incubated with anti-c-kit primary antibody in a humid chamber at 4 °C overnight, rinsed with PBS, and sequentially incubated at room temperature with biotinylated antirabbit immunoglobulin (1 : 200; Vector, Burlingame, CA, USA), streptavidin-peroxidase complex (Dako Cytomation, Glostrup, Denmark), and finally with 3,3′-diaminobenzidine tetrahydrocloride enhanced with nickel chloride (Sigma-Aldrich). Specimens were mounted with DPX® mountant for histology (Fluka, Buchs, Switzerland), examined by light microscope, and representative photomicrographs were taken by DFC480 digital camera (Leica, Cambridge, UK). To obtain the best tissue orientation for analysing the morphology and distribution of c-kit immunoreactive ICC, only colonic cross-sections were examined. Negative controls to assess non-specific staining were obtained by omitting primary antibodies or substituting them with normal rabbit serum (1 : 100). Positive control staining was detected in mast cells that are known to constitutively express c-kit receptors (see Fig. 6, panel A in the Results section).[26]

Whole mounts preparations of myenteric plexus (WM-MP) for immunofluorescence staining were obtained by removing the mucosa, submucosa and circular muscle layer from each sample. WM-MP preparations were incubated in 10% normal donkey serum in PBS containing 1% Triton® X-100 for 1 h at room temperature to block non-specific binding. WM-MP preparations were washed three times with PBS (twice for 10 min and once for 30 min) and incubated at room temperature for 3 days with anti-β_3-adrenoceptor antibody and anti-c-kit antibodies or anti-PGP 9.5 antibody diluted in 5% donkey serum in 0.5% Triton® X-100 (Sigma-Aldrich) in PBS. After three washings in PBS, secondary antibodies Alexa Fluor® 555 donkey antigoat IgG (1 : 1200, Molecular Probes), Alexa Fluor® 488 donkey antirabbit IgG (1 : 600, Molecular Probes) or Alexa Fluor® 488 donkey anti-mouse IgG (1 : 1000, Molecular Probes) were applied for 2 h at room temperature. After three further washings in PBS, WM-MP preparations were mounted with Mowiol® 4-88 reagent (Calbiochem) and examined by fluorescence microscope (ECLIPSE 90i; Nikon Instruments) and representative photomicrographs were taken by DS-5M digital camera (Nikon Instruments). To verify the specificity of immunohistochemical detections, the following control experiments

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

were performed: omission of the primary antibody and tissue section pre-adsorption by incubation of the β_3-adrenoceptor antibody with the β_3-adrenoceptor blocking peptide (2 μg mL^{-1}; sc-1473P, Santa Cruz Biotechnology).

Statistical analysis

Results are expressed as mean values ± standard error mean (SEM). Statistical analysis was performed using analysis of variance (one-way or two-way, as appropriate, with the Bonferroni's correction for multiple comparisons). A P-value < 0.05 was considered significant; n refers to the number of animals used for each experiment (n = 8–16). Calculations were performed using GraphPad Prism™ (version 4.0; GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Assessment of colitis

2,4-Dinitrobenzene sulphonic acid dose-dependently increased inflammatory damage, as indicated by macroscopic and microscopic damage score and MPO activity (Fig. 1A). Both 15 and 30 mg per rat of DNBS induced severe inflammation (of comparable magnitude on the three parameters under scrutiny), but with different spontaneous mortality rates (0% and 17% respectively). Thus, we selected 15 mg of DNBS per rat for all subsequent experiments.

Time-course experiments showed that all parameters under scrutiny peaked on day 6 postcolitis induction, with a trend to decrease on day 10 (Fig. 1B). Myeloperoxidase activity, as expected, achieved high values as early as day 3 (because of early neutrophil infiltration after induction of colitis) and peaked on day 6. Thus, day 6 was selected to kill the animals. At day 6 after DNBS administration, the distal colon was thickened and ulcerated with evident areas of transmural inflammation. Adhesions were often present and the bowel was occasionally dilated and there was a greater than fivefold increase in macroscopic damage score (Table 1). Colitis was characterized by a massive granulocyte infiltrate extending throughout the mucosa and submucosa, often involving the muscularis propria, which invariably appeared thickened (Fig. 2B) and by a 13-fold increase in microscopic damage score over the non-inflamed control animals (Table 1). 2,4-Dinitrobenzene sulphonic acid treatment induced a 27-fold increase in MPO activity (Table 1), a more than threefold increase in TNF-α tissue levels, a fivefold and 1.5-fold increase in IL-1 β and IL-6 tissue levels, respectively, compared with non-inflamed rats (Table 2). With intrarectal administration of DNBS, body weight gain was significantly reduced with respect to non-inflamed controls (Fig. 3).

Effect of SR58611A in rats without colitis

Administration of SR58611A *per se* at the highest dose in non-inflamed rats had no significant effects on macroscopic and microscopic scores or colonic MPO activity (Table 1). Body weight gain of non-inflamed rats was 26.40 ± 1.43% during the period of observation; administration of SR58611A (10 mg kg^{-1} orally) in non-inflamed rats had no significant effect on body weight gain (Fig. 3).

Effect of SR58611A on DNBS-induced colitis

Treatment with SR58611A, at the doses of 1, 3 and 10 mg kg^{-1}, significantly reduced the impairment in body weight gain induced by colitis (Fig. 3) and produced a significant (approximately 40%) reduction in macroscopic damage (Table 1). Moreover, treatment with SR58611A dose-dependently decreased the microscopic damage scores (Table 1, Fig. 2C–D) and reduced the inflammatory cell infiltrate, although statistical significance was achieved only at the doses of 3 and

Table 1 Effect of SR58611A in non-inflamed and inflamed rats

	Non-inflamed (intrarectal vehicle)		Inflamed (intrarectal DNBS 15 mg per rat)			
Oral	SR58611A vehicle	SR58611A 10 mg kg^{-1}	SR58611A vehicle	SR58611A 1 mg kg^{-1}	SR58611A 3 mg kg^{-1}	SR58611A 10 mg kg^{-1}
Macroscopic score	1.10 ± 0.10	1.13 ± 0.13	6.33 ± 0.73*	3.25 ± 0.45†	4.00 ± 0.18‡	3.46 ± 0.39†
Microscopic score	0.40 ± 0.16	0.88 ± 0.23	5.25 ± 0.37*	4.50 ± 0.71	3.13 ± 0.46‡	3.00 ± 0.45†
MPO activity	1.80 ± 0.25	1.40 ± 0.20	48.85 ± 9.11*	69.94 ± 18.14	37.27 ± 5.98	25.61 ± 5.04‡

Data are mean values (±SEM); n = 8–16 per group; DNBS, 2,4-dinitrobenzene sulphonic acid; MPO, myeloperoxidase. *P < 0.001 *vs* intrarectal vehicle; $^{\dagger}P$ < 0.01 *vs* intrarectal DNBS + SR58611A vehicle; $^{\ddagger}P$ < 0.05 *vs* intrarectal DNBS + SR58611A vehicle.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

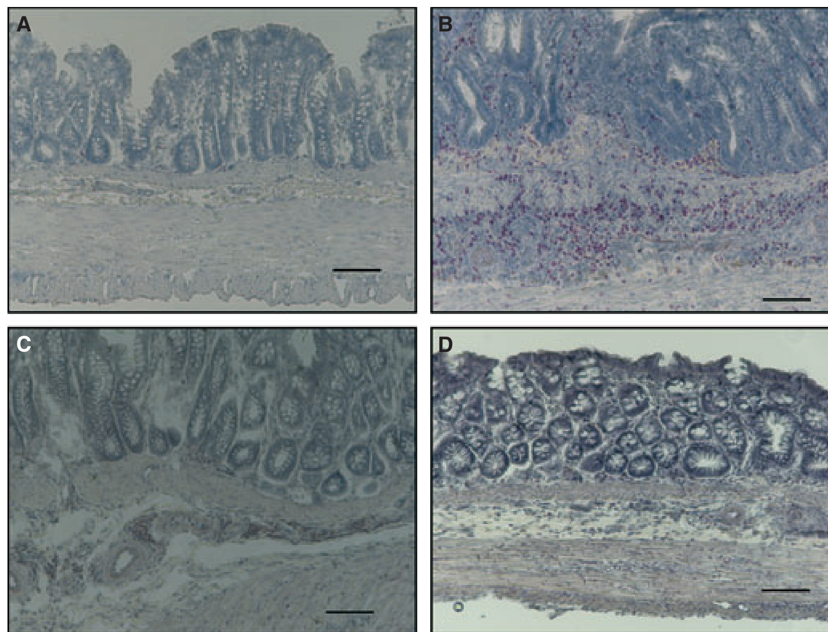

Figure 2 Representative examples of cross-sections of distal colon from a control rat (A) (intrarectal vehicle + SR58611A vehicle orally) and (B) from an inflamed rat (intrarectal DNBS 15 mg in 50% ethanol + SR58611A vehicle orally). Note the dramatic loss of mucosal architecture with goblet cell depletion and the massive granulocyte infiltrate extending throughout the mucosa and submucosa, also involving the muscularis propria, which appears thickened (H&E). In C and D, cross-sections of distal colon from an inflamed rat treated with SR58611A 3 mg kg^{-1} (C) or 10 mg kg^{-1} (D). Note the reduction of mucosal architecture loss, the decrease in ulcerations and a reduction in muscle thickening. Scale bar: 100 μm.

Table 2 Effect of SR58611A on cytokine tissue levels

	Intrarectal vehicle	Intrarectal DNBS (15 mg per rat)	
Oral	SR58611A vehicle	SR58611A vehicle	SR58611A 10 mg kg^{-1}
TNF-α level	0.103 ± 0.033	0.397 ± 0.053*	0.227 ± 0.038†
IL-1β level	1.59 ± 0.37	7.66 ± 2.84*	2.15 ± 0.43†
IL-6 level	5.70 ± 1.34	8.23 ± 1.11	3.97 ± 1.20†

Data are mean values (±SEM); n = 4–8 per group. DNBS, 2,4-dinitrobenzene sulphonic acid; TNF-α, tumour necrosis factor-α; IL, interleukin. *P < 0.01 *vs* intrarectal vehicle; †P < 0.05 *vs* intrarectal DNBS + SR58611A vehicle.

10 mg kg^{-1}. Myeloperoxidase activity showed a trend towards decreased levels at the dose of 3 mg kg^{-1}, and statistical significance was achieved at the dose of 10 mg kg^{-1} (Table 1). SR58611A also decreased TNF-α, IL-1β and IL-6 tissue levels (Table 2).

RT-PCR analysis of β_3-adrenoceptor expression

Reverse transcription–polymerase chain reaction assay of colonic tissues revealed the expression of β_3-adrenoceptor mRNA in mucosal/submucosal and muscular layers of proximal and distal colon of non-inflamed and inflamed animals. The densitometric analysis of the amplified cDNA bands showed a similar expression level of β_3-adrenoceptor mRNA in non-inflamed rats, with no significant differences between distal and proximal colonic portions, as well as between mucosal/submucosal and muscular layers (Fig. 4, panels A and B). The induction of colitis significantly reduced the β_3-adrenoceptor mRNA expression in the mucosal/submucosal layer of distal colon, but did not significantly affect the expression of β_3-adrenoceptor mRNA in distal and proximal colonic muscle layers, and

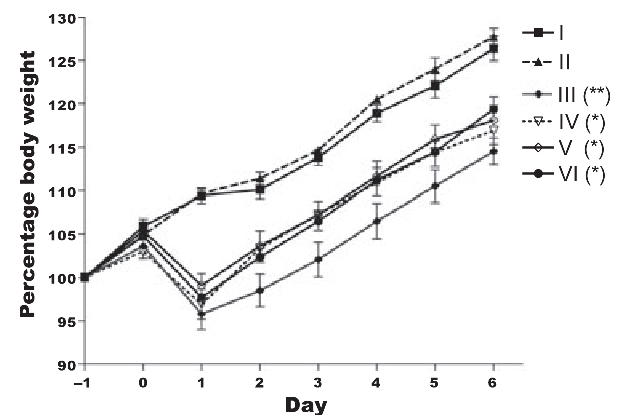

Figure 3 Body weight change in the different experimental groups. Data are expressed as mean values ± SEM. n = 8–16 rats per group. Group I: intrarectal vehicle and SR58611A vehicle orally; Group II: intrarectal vehicle and SR58611A 10 mg kg^{-1} orally; Group III: intrarectal DNBS 15 mg and SR58611A vehicle orally; Group IV: intrarectal DNBS 15 mg and SR58611A 1 mg kg^{-1} orally; Group V: intrarectal DNBS 15 mg and SR58611A 3 mg kg^{-1} orally; Group VI: intrarectal DNBS 15 mg and SR58611A 10 mg kg^{-1} orally. Statistical analysis was performed with two-way analysis of variance; *P < 0.01 *vs* Group I; **P < 0.01 *vs* Group III.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

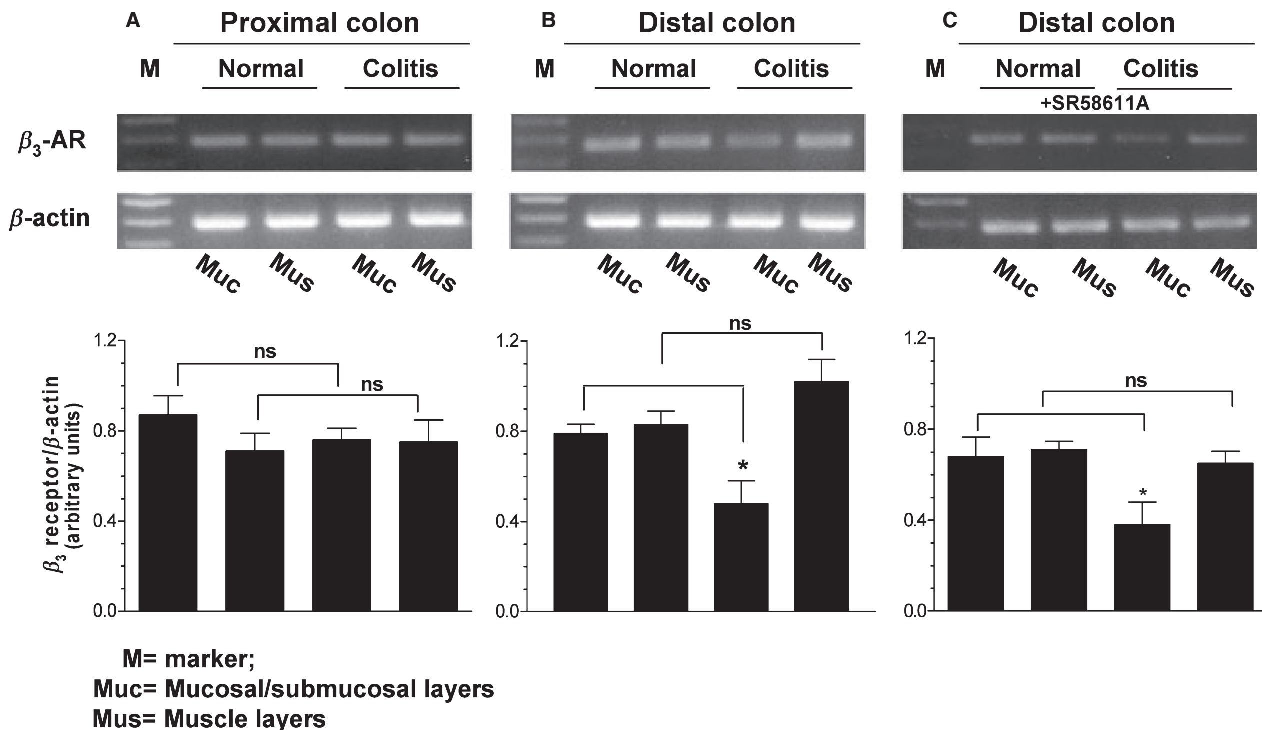

Figure 4 RT-PCR analysis of β_3-adrenoceptor (β_3-AR) and β-actin mRNA expression in mucosal/submucosal (Muc) and muscular (Mus) layers of proximal (A) or distal colon (B and C) isolated from animals in the absence (vehicle) or in the presence of DNBS-induced colitis (DNBS). Animals in panel C were treated with SR58611A (10 mg kg^{-1} orally). Each panel displays two representative agarose gels, referring to the amplification of β_3-adrenoceptor (38 cycles) and β-actin (27 cycles) cDNAs, and a column graph referring to the densitometric analysis of β_3-adrenoceptor cDNA bands normalized to the expression of β-actin. M = size markers. Each column represents the mean of five experiments ± SEM (vertical bars); $^{*}P < 0.05$ *vs* the respective values obtained in normal animals.

in mucosal/submucosal layer of proximal colon (Fig. 4, panels A and B). Treatment with SR58611A 10 mg kg^{-1} did not affect β_3-adrenoceptor mRNA expression either in the absence or in the presence of DNBS-induced colitis (Fig. 4C).

Immunohistochemistry

Immunohistochemical analysis revealed the presence of β_3-adrenoceptors in submucosal blood vessels and within the neuromuscular compartment (Fig. 5). In the muscular layer, β_3-adrenoceptor immunopositivity was mainly localized in the circular muscle layer close to the submucosa and in the myenteric plexus; scarce and scattered immunopositive cells were also detectable in the mucosa (Fig. 5). Incubation of anti-β_3-adrenoceptor primary antibody with β_3-adrenoceptor blocking peptide abolished immunoreactivity, indicating specific immunostaining.

C-kit positive cells were detectable throughout the neuromuscular layer and were identified as ICCs (Fig. 6A,B). In line with previous reports,[27] immunohistochemistry showed the presence of two major populations of c-kit immunoreactive cells: one along the submucosal border of the circular muscle, the submucosal ICC (ICC-SM; Fig. 6A), and one between the circular and longitudinal muscular layers near the myenteric plexus (ICC-MP; Fig. 6B). Intramural spindle shaped cells were observed within the circular muscle layer (ICC-CM; Fig. 6A).

Whole mounts preparations of myenteric plexus stained for β_3-adrenoceptors showed immmunolabelled nerve fibres within the neuromuscular layer; c-kit positive cells were identified within the myenteric plexus, in very close apposition to β_3-immunoreactive nerve terminals, indicating that β_3-immunoreactivity could be on nerve fibres innervating ICCs (Fig. 7C) as demonstrated on the PGP 9.5-positive nerve fibres in Fig. 8F. In addition, β_3-adrenoceptor immunoreactivity was identified in perikarya of a subset of PGP 9.5 labelled myenteric neurons (Fig. 8C).

In inflamed tissues, β_3-adrenoceptor immunoreactivity was decreased in the submucosa, in the region of the circular muscle layer close to the

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

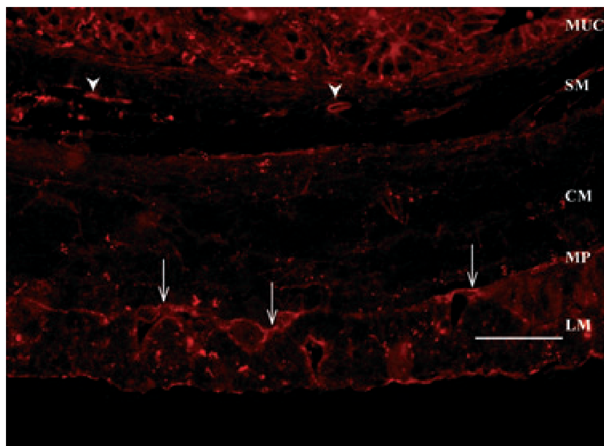

Figure 5 Representative areas from cross-sections of normal rat distal colon showing β_3-adrenoceptor immunoreactivity. β_3-Adrenoceptor immunoreactivity was mainly observed in submucosal blood vessels (arrowheads) and within the muscular layers of the distal colon, particularly at the submucosal border of circular muscle layer and in the myenteric plexus region (arrows). MUC, mucosa; SM, submucosa; CM, circular muscle; MP, myenteric plexus; LM, longitudinal muscle. Scale bar = 50 μm.

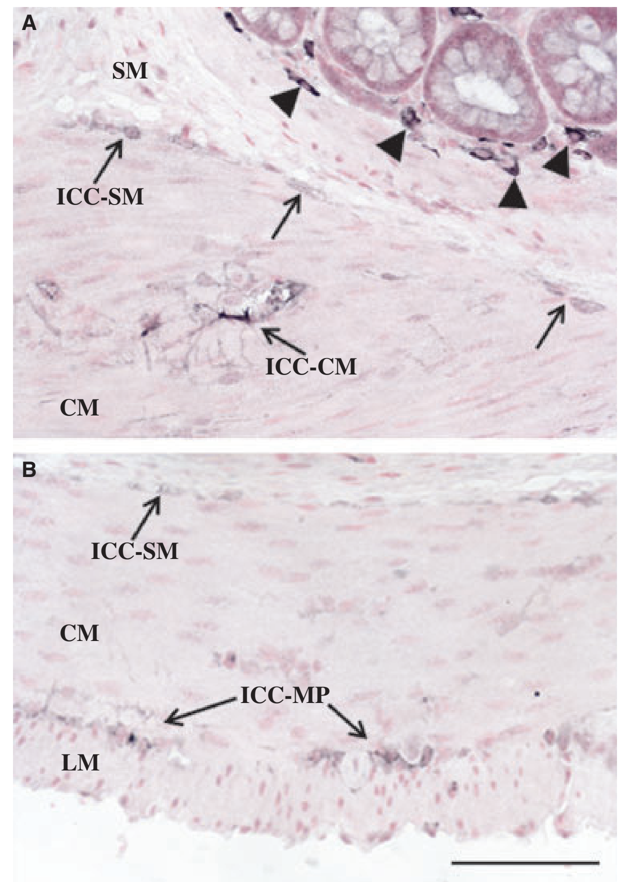

Figure 6 Representative area from cross-sections of normal rat distal colon showing c-kit immunoreactivity. Arrows and arrowheads indicate c-kit positive interstitial cells of Cajal (ICCs) and mucosal mast cells respectively. Panel A shows the presence of ICC along the submucosal border (ICC-SM) of circular muscle (CM) and within the CM (ICC-CM) layer; an ICC-CM with its dendritic extensions is clearly evident. Panel B: c-kit positive cells were also identified between the CM and longitudinal muscle (LM) layer along the myenteric plexus (ICC-MP). SM, submucosa; CM, circular muscle; LM, longitudinal muscle. Scale bar = 100 μm.

submucosal layer (not shown) and in the myenteric plexus (Fig. 7D). Likewise, only faint c-kit positivity was observed at the level of ICC-MP, accompanied by a disorganization of the ICC network (Fig. 7E). This reduction of c-kit staining was particularly evident in areas of macroscopic damage. This finding supports the hypothesis that during experimental colitis the ICC network is impaired and c-kit immunoreactivity is decreased within the colonic wall, as recently observed by Kinoshita *et al.*[28] In addition, a decreased β_3-adrenoceptor immunolabelling was found in myenteric neurons of WM-MP from DNBS-treated rats (data not shown).

After treatment with SR58611A at the dose of 10 mg kg^{-1}, β_3-adrenoceptor and c-kit immunoreactivity were similar to non-inflamed controls (Fig. 7G,H).

DISCUSSION

The present study shows that: (i) the β_3-adrenoceptor agonist SR58611A, at the dose of 10 mg kg^{-1}, exerts a protective effect on DNBS-induced colitis in rats by counteracting the increase in macroscopic and microscopic damage score, MPO activity and cytokine tissue levels; (ii) β_3-adrenoceptor expression was found not only, as expected, on the submucosal blood vessels, but also within the neuromuscular layer of the distal colon, on myenteric neurons and nerve fibres in close vicinity of ICCs.

Our data, showing decreased expression of β_3-adrenoceptors during colitis within the myenteric plexus and in the neuromuscular layer are consistent with the downregulation of the inhibitory β_3-adrenergic control observed by Zhao *et al.*[12] in the same species during experimental colitis. The decreased β_3-adrenoceptor expression (present study) and the reduced evoked noradrenaline release from enteric nerves[13] observed during colitis suggest that a decrease in the inhibitory β_3-adrenergic regulation may be involved in inflammation. Thus, the β_3-adrenoceptor stimulation with the selective β_3-adrenoceptor agonist SR58611A may counteract intestinal inflammation in

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

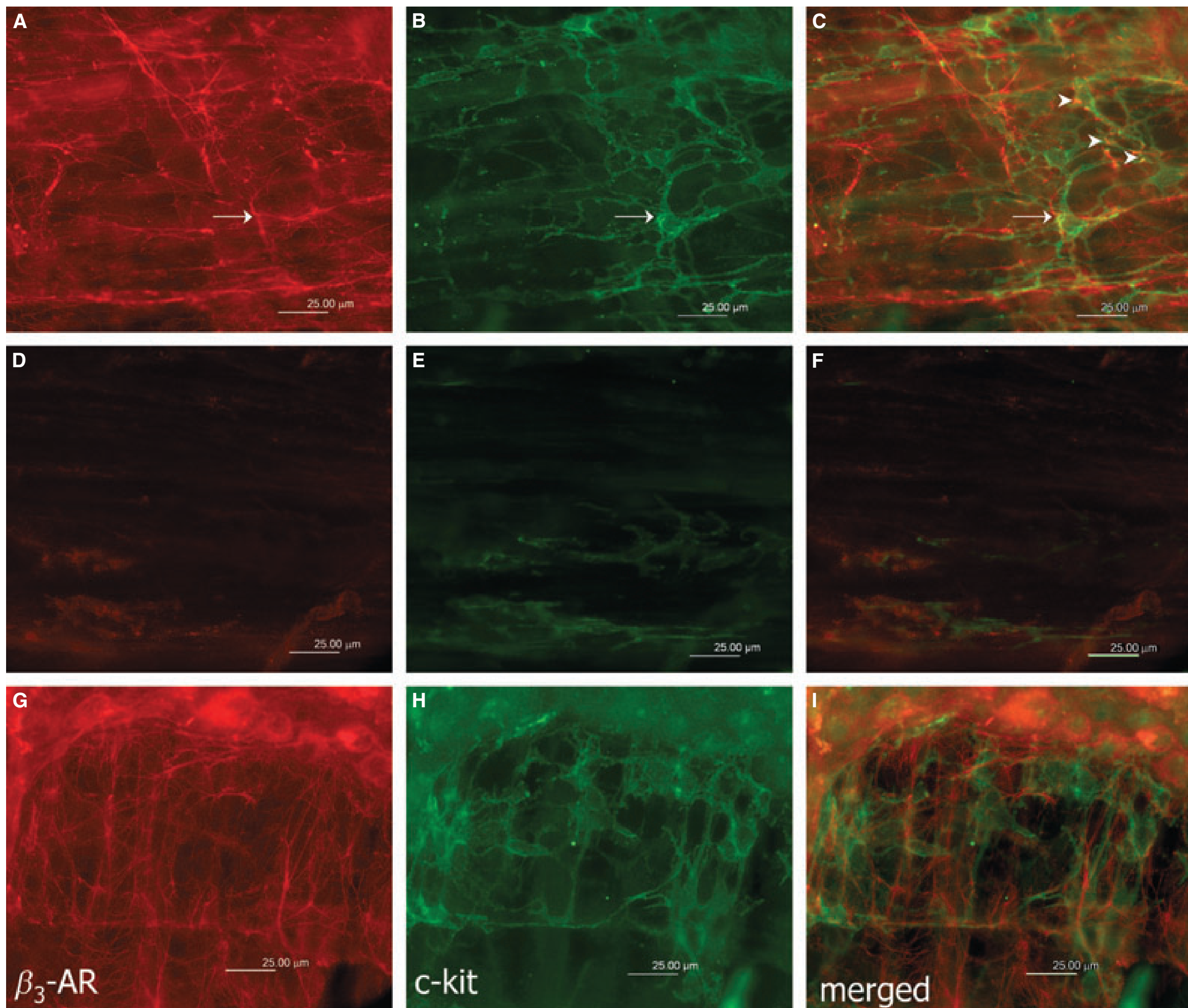

Figure 7 β_3-Adrenoceptor (red, panels A, D and G) and c-kit (green, panels B, E and H) immunoreactivity in distal colonic WM preparations of myenteric plexus from a normal rat (A,B), a rat with colitis (D,E) and a rat with colitis treated with SR58611A 10 mg kg^{-1} (G,H). In panels C, F and I, the simultaneous localization of β_3-adrenoceptor and c-kit immunoreactivities is visualized in a normal rat (C), a rat with colitis (F) and a rat with colitis treated with SR58611A 10 mg kg^{-1} (I). Panels A–C: (A) β_3-adrenoceptor immunoreactivity was observed in structures at the level of myenteric plexus region. Double staining experiments revealed the presence of β_3-adrenoceptor in spot-like points (arrowheads, C) in close proximity to (B) c-kit positive cells (arrows, panels A–C). Furthermore, compared to non-inflamed rats, tissues from rats with colitis showed decreased β_3-adrenoceptor immunoreactivity (D). Inflammation was also associated with a decreased c-kit immunoreactivity along the myenteric plexus (E). Treatment with SR58611A restored β_3-adrenoceptor (G) and c-kit (H) immunoreactivity. Scale bar = 25 μm.

part by restoring the loss in inhibitory adrenergic control caused by inflammatory stimuli.

An important finding in our study was the identification of β_3-adrenoceptor immunoreactivity in a subpopulation of myenteric neurons as well as in close vicinity to ICCs (i.e. ICC-MP) within the myenteric plexus. The former observation is in line with a recent study carried out in the human colon, which detected β_3-adrenoceptor immunoreactivity in a majority of ChAT-positive myenteric and submucosal neurons.[6] Our data, showing β_3 immunoreactivity in close proximity to ICC-MP and the observations that ICCs are in close proximity of nitric oxide synthase and vesicular acetylcholine transporter immunoreactive nerve terminals,[29–31] suggest a possible cross-talk between neurons and ICCs.

In our experiments, inflammation was characterized by decreased β_3-adrenoceptor expression according to

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

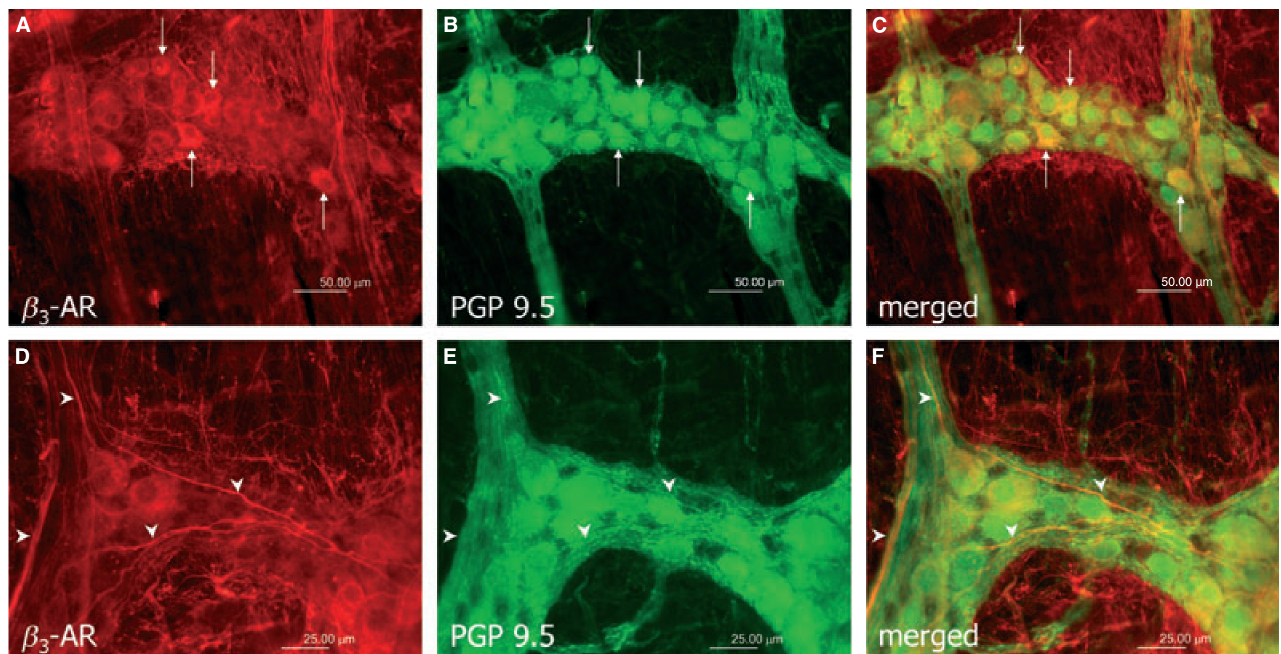

Figure 8 β_3-Adrenoceptor (red, panels A and D), PGP 9.5 (green, panels B and E) and co-localization of β_3-adrenoceptor and PGP 9.5 immunoreactivities (yellow, panels C and F) in distal colonic whole mount preparations of myenteric plexus from a normal rat. Neuronal perikarya and nerve fibres are indicated by arrows (A–C) and arrowheads (D–F), respectively. Scale bars = 50 and 25 μm in panels A–C and D–F respectively.

immunohistochemistry (Fig. 7), whereas reduced β_3-adrenoceptor mRNA expression was found only in the mucosal/submucosal layer of the inflamed distal colon, but not in the neuromuscular compartment (Fig. 4). There are several explanations for this discrepancy: (i) the inflammatory status of colonic tissues: indeed, G-protein-coupled receptors are subject to complex post-transcriptional regulation and inflammation can alter such mechanisms, leading to different patterns of mRNA and protein expression. In keeping with this view, α_1-adrenoceptor expression in rat liver during sepsis varied by 29% and 55% upon evaluation of mRNA and protein levels, respectively;[32] (ii) localization of β_3-positivity undergoing downregulation during inflammation: we found reduced positivity in the submucosa (no discrepancy between the results obtained by the two techniques) and, according to immunohistochemistry, at the border between the circular muscle layer and the submucosa as well as in the myenteric plexus. This observation was not confirmed by RT-PCR possibly because, when separating the mucosal/submucosal layer from the muscular layer, β_3-adrenoceptor expressing cells were cleaved together with submucosal tissue. In line with this hypothesis, Plujà *et al.*[23] reported a similar experience, since several unidentified dead cells (possibly including β_3-expressing cells) with a broken cytoplasmatic membrane remained at the submucosal border of the circular muscle when the submucosa was stripped off.

There is substantial evidence showing that experimental inflammation evokes ICC changes in the deep muscular plexus (ICC-DMP) of the small bowel characterized by loss of c-kit immunoreactivity.[24,33,34] In our study, DNBS-induced inflammation produced a decrease in c-kit-positive ICCs, and treatment with SR58611A, at the dose of 10 mg kg^{-1}, restored both c-kit immunolabeling and β_3-adrenoceptor immunoreactivity. Our data, showing a SR58611A-induced reduction in TNF-α, IL-1β and IL-6 tissue levels, are consistent with the hypothesis of a β_3-adrenoceptor-mediated inhibition of pro-inflammatory cytokine production. Notably, IL-1, IL-6 and TNF-α can affect neurotransmitter release from myenteric neurons,[35] while IL-1β can inhibit acetylcholine and noradrenaline release from the myenteric plexus.[36]

Besides a neurally mediated effect, one could also hypothesize that β_3-adrenoceptors could exert a direct control on immune cell function. It is well known that macrophages express α_2- and β-adrenoceptors,[37,38] which can exert an excitatory or inhibitory effect on inflammatory cells respectively. In previous

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

studies, the administration of β-adrenoceptor agonists, such as isoprotenerol or clenbuterol, suppressed lipopolysaccaride-induced increase in TNF-α and IL-6 production both *in vitro* and *in vivo*,[39] confirming a role of β-adrenoceptors in the modulation of cytokine production, probably through an increase in cAMP levels leading to a reduced release of pro-inflammatory mediators or an induction of anti-inflammatory mediators.[40] Nevertheless, evidence of β_3-adrenoceptors on immune cells is lacking.

Although the effects of SR58611A suggest a role of β_3-adrenoceptors in modulating the immune/inflammatory response, possibly through a neurally-mediated mechanism, we cannot rule out other mechanisms that may participate in colonic protection.

Recently, Cellek *et al.*[6] showed that activation of β_3-adrenoceptor with β_3-adrenergic agonists lead to an inhibition of cholinergic contractions and evoked somatostatine release, resulting in a decrease of intestinal motility and secretion and inducing analgesia.

β_3-Adrenoceptor agonist administration confers gastroprotection in several models of gastric ulcer,[41,42] an effect that may be due to increased blood flow by vasodilatation and/or relaxation of the *muscularis externa* mediated by β_3-adrenoceptors. Indeed, an improved blood flow and inhibition of colonic motility might contribute to the protective action of SR58611A in DNBS-induced colitis.

In conclusion, this study shows that treatment with a selective β_3-adrenoceptor agonist ameliorates DNBS-induced colitis in rats and downregulates the expression of inflammatory cytokines. In this context, the finding of β_3-adrenoceptors on myenteric neurons and in nerve fibres bears implications on the role of these receptors as a potential therapeutic target for gut inflammatory disease.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the University of Bologna (years 2003 and 2004) and the Italian Ministry of Education, University and Research (COFIN 2002 Project 2002052573).

REFERENCES

1 De Ponti F, Gibelli G, Croci T, Arcidiaco M, Crema F, Manara L. Functional evidence of atypical beta 3-adrenoceptors in the human colon using the beta 3-selective adrenoceptor antagonist, SR 59230A. *Br J Pharmacol* 1996; **117**: 1374–6.

2 Bianchetti A, Manara L. *In vitro* inhibition of intestinal motility by phenylethanolaminotetralines: evidence of atypical beta-adrenoceptors in rat colon. *Br J Pharmacol* 1990; **100**: 831–9.

3 Kelly J, Houston G. Beta 3-adrenoceptors mediating relaxation of the oesophageal tunica muscularis mucosae and distal colon in the rat: comparative pharmacology and their desensitization by BRL 37344. *J Auton Pharmacol* 1996; **16**: 205–11.

4 De Ponti F, Modini C, Gibelli G, Crema F, Frigo G. Atypical beta-adrenoceptors mediating relaxation in the human colon: functional evidence for beta3-rather than beta4-adrenoceptors. *Pharmacol Res* 1999; **39**: 345–8.

5 Anthony A, Schepelmann S, Guillaume JL *et al.* Localization of the beta(beta)3-adrenoceptor in the human gastrointestinal tract: an immunohistochemical study. *Aliment Pharmacol Ther* 1998; **12**: 519–25.

6 Cellek S, Thangiah R, Bassil AK *et al.* Demonstration of functional neuronal beta(3)-adrenoceptors within the enteric nervous system. *Gastroenterology* 2007; **133**: 175–83.

7 Anthony A, Bahl AK, Oakley IG *et al.* The beta 3-adrenoceptor agonist CL316243 prevents indomethacin-induced jejunal ulceration in the rat by reversing early villous shortening. *J Pathol* 1996; **179**: 340–6.

8 Coruzzi G, Bertaccini G. The beta3-adrenoceptor agonist SR58611A inhibits gastric acid secretion in the conscious cat. *Naunyn Schmiedebergs Arch Pharmacol* 1997; **356**: 263–5.

9 Bertini S, Coruzzi G, Intorre L, Soldani G. Effects of beta3-adrenoceptor agonist SR 58611A on gastric acid secretion and histamine release in the dog: comparison with ritodrine. *Gen Pharmacol* 1998; **31**: 625–31.

10 Kelly D, Piasecki C, Anthony A, Dhillon AP, Pounder RE, Wakefield AJ. Reversal and protection against indomethacin-induced blood stasis and mucosal damage in the rat jejunum by a beta3-adrenoceptor agonist. *Aliment Pharmacol Ther* 1998; **12**: 1121–9.

11 Vinay HK, Paul A, Goswami SS, Santani D. Effect of SR 58611A, a beta-3 receptor agonist, against experimental gastro-duodenal ulcers. *Indian J Physiol Pharmacol* 2002; **46**: 36–44.

12 Zhao A, Bossone C, Pineiro-Carrero V, Shea-Donohue T. Colitis-induced alterations in adrenergic control of circular smooth muscle *in vitro* in rats. *J Pharmacol Exp Ther* 2001; **299**: 768–74.

13 Blandizzi C, Fornai M, Colucci R *et al.* Altered prejunctional modulation of intestinal cholinergic and noradrenergic pathways by alpha2-adrenoceptors in the presence of experimental colitis. *Br J Pharmacol* 2003; **139**: 309–20.

14 Manara L, Croci T, Landi M. Beta 3-adrenoceptors and intestinal motility. *Fundam Clin Pharmacol* 1995; **9**: 332–42.

15 Giudice A, Croci T, Bianchetti A, Manara L. Inhibition of rat colonic motility and cardiovascular effects of new gut-specific beta-adrenergic phenylethanolaminotetralines. *Life Sci* 1989; **44**: 1411–7.

16 Montastruc JL, Verwaerde P, Pelat M *et al.* Peripheral cardiovascular actions of SR 58611 A, a beta 3-adrenoceptor agonist, in the dog: lack of central effect. *Fundam Clin Pharmacol* 1999; **13**: 180–6.

17 Elson CO, Sartor RB, Tennyson GS, Riddell RH. Experimental models of inflammatory bowel disease. *Gastroenterology* 1995; **109**: 1344–67.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

18 Wallace JL, Le T, Carter L, Appleyard CB, Beck PL. Hapten-induced chronic colitis in the rat: alternatives to trinitrobenzene sulfonic acid. *J Pharmacol Toxicol Methods* 1995; **33**: 237–9.
19 De Ponti F, Gibelli G, Crema F, Lecchini S. Functional evidence for the presence of beta 3-adrenoceptors in the guinea pig common bile duct and colon. *Pharmacology* 1995; **51**: 288–97.
20 Sturiale S, Barbara G, Qiu B *et al.* Neutral endopeptidase (EC 3.4.24.11) terminates colitis by degrading substance P. *Proc Natl Acad Sci USA* 1999; **96**: 11653–8.
21 Boughton-Smith NK, Wallace JL, Whittle BJ. Relationship between arachidonic acid metabolism, myeloperoxidase activity and leukocyte infiltration in a rat model of inflammatory bowel disease. *Agents Actions* 1988; **25**: 115–23.
22 Jacobson K, McHugh K, Collins SM. Experimental colitis alters myenteric nerve function at inflamed and noninflamed sites in the rat. *Gastroenterology* 1995; **109**: 718–22.
23 Pluja L, Alberti E, Fernandez E, Mikkelsen HB, Thuneberg L, Jimenez M. Evidence supporting presence of two pacemakers in rat colon. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G255–66.
24 Faussone-Pellegrini MS, Gay J, Vannucchi MG, Corsani L, Fioramonti J. Alterations of neurokinin receptors and interstitial cells of Cajal during and after jejunal inflammation induced by Nippostrongylus brasiliensis in the rat. *Neurogastroenterol Motil* 2002; **14**: 83–95.
25 Bernardini N, Colucci R, Mattii L *et al.* Constitutive expression of cyclooxygenase-2 in the neuromuscular compartment of normal human colon. *Neurogastroenterol Motil* 2006; **18**: 654–62.
26 Reber L, Da Silva CA, Frossard N. Stem cell factor and its receptor c-Kit as targets for inflammatory diseases. *Eur J Pharmacol* 2006; **533**: 327–40.
27 Vanderwinden JM, Rumessen JJ, Bernex F, Schiffmann SN, Panthier JJ. Distribution and ultrastructure of interstitial cells of Cajal in the mouse colon, using antibodies to Kit and Kit(W-lacZ) mice. *Cell Tissue Res* 2000; **302**: 155–70.
28 Kinoshita K, Horiguchi K, Fujisawa M *et al.* Possible involvement of muscularis resident macrophages in impairment of interstitial cells of Cajal and myenteric nerve systems in rat models of TNBS-induced colitis. *Histochem Cell Biol* 2007; **127**: 41–53.
29 Ward SM, Beckett EA, Wang X, Baker F, Khoyi M, Sanders KM. Interstitial cells of Cajal mediate cholinergic neurotransmission from enteric motor neurons. *J Neurosci* 2000; **20**: 1393–403.
30 Wang XY, Sanders KM, Ward SM. Relationship between interstitial cells of Cajal and enteric motor neurons in the murine proximal colon. *Cell Tissue Res* 2000; **302**: 331–42.
31 Ward SM, Sanders KM, Hirst GD. Role of interstitial cells of Cajal in neural control of gastrointestinal smooth muscles. *Neurogastroenterol Motil* 2004; **16**(Suppl. 1): 112–7.
32 Dong LW, Yang J, Tong LJ, Tang C, Liu MS. Transcriptional regulation of alpha-1adrenoceptor gene in the rat liver during different phases of sepsis. *Biochim Biophys Acta* 1999; **1453**: 207–15.
33 Wang XY, Berezin I, Mikkelsen HB *et al.* Pathology of interstitial cells of Cajal in relation to inflammation revealed by ultrastructure but not immunohistochemistry. *Am J Pathol* 2002; **160**: 1529–40.
34 Wang XY, Vannucchi MG, Nieuwmeyer F, Ye J, Faussone-Pellegrini MS, Huizinga JD. Changes in interstitial cells of Cajal at the deep muscular plexus are associated with loss of distention-induced burst-type muscle activity in mice infected by Trichinella spiralis. *Am J Pathol* 2005; **167**: 437–53.
35 Collins SM. The immunomodulation of enteric neuromuscular function: implications for motility and inflammatory disorders. *Gastroenterology* 1996; **111**: 1683–99.
36 Hurst SM, Stanisz AM, Sharkey KA, Collins SM. Interleukin 1 beta-induced increase in substance P in rat myenteric plexus. *Gastroenterology* 1993; **105**: 1754–60.
37 Abrass CK, O'Connor SW, Scarpace PJ, Abrass IB. Characterization of the beta-adrenergic receptor of the rat peritoneal macrophage. *J Immunol* 1985; **135**: 1338–41.
38 Spengler RN, Allen RM, Remick DG, Strieter RM, Kunkel SL. Stimulation of alpha-adrenergic receptor augments the production of macrophage-derived tumor necrosis factor. *J Immunol* 1990; **145**: 1430–4.
39 Izeboud CA, Monshouwer M, van Miert AS, Witkamp RF. The beta-adrenoceptor agonist clenbuterol is a potent inhibitor of the LPS-induced production of TNF-alpha and IL-6 *in vitro* and *in vivo*. *Inflamm Res* 1999; **48**: 497–502.
40 Severn A, Rapson NT, Hunter CA, Liew FY. Regulation of tumor necrosis factor production by adrenaline and beta-adrenergic agonists. *J Immunol* 1992; **148**: 3441–5.
41 Sevak R, Paul A, Goswami S, Santani D. Gastroprotective effect of beta3 adrenoreceptor agonists ZD 7114 and CGP 12177A in rats. *Pharmacol Res* 2002; **46**: 351–6.
42 Kuratani K, Kodama H, Yamaguchi I. Enhancement of gastric mucosal blood flow by beta-3 adrenergic agonists prevents indomethacin-induced antral ulcer in the rat. *J Pharmacol Exp Ther* 1994; **270**: 559–65.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd