RESEARCH ARTICLE

Experimental colitis in rats induces *de novo* synthesis of cytokines at distant intestinal sites: role of capsaicin-sensitive primary afferent fibers

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ABSTRACT. ***Introduction:*** **Increased levels of pro- and anti-inflammatory cytokines were observed in various segments of histologically-intact small intestine in animal models of acute and chronic colitis. Whether these cytokines are produced locally or spread from the inflamed colon is not known. In addition, the role of gut innervation in this upregulation is not fully understood.** ***Aims:*** **To examine whether cytokines are produced *de novo* in the small intestine in two rat models of colitis; and to investigate the role of capsaicin-sensitive primary afferents in the synthesis of these inflammatory cytokines.** ***Methods:*** **Colitis was induced by rectal instillation of iodoacetamide (IA) or trinitrobenzene sulphonic acid (TNBS) in adult Sprague-Dawley rats. Using reverse transcriptase (RT) and real-time PCR, TNF-α, and IL-10 mRNA expression was measured in mucosal scrapings of the duodenum, jejunum, ileum and colon at different time intervals after induction of colitis. Capsaicin-sensitive primary afferents (CSPA) were ablated using subcutaneous injections of capsaicin at time 0, 8 and 32 h, and the experiment was repeated at specific time intervals to detect any effect on cytokines expression.** ***Results:*** **TNF-α mRNA expression increased by 3-40 times in the different intestinal segments ($p<0.05$ to $p<0.001$), 48h after IA-induced colitis. CSPA ablation completely inhibited this upregulation in the small intestine, but not in the colon. Similar results were obtained in TNBS-induced colitis at 24 h. Intestinal IL-10 mRNA expression significantly decreased at 12 h and then increased by 6-43 times ($p<0.05$ to $p<0.001$) 48h after IA administration. This increase was abolished in rats subjected to CSPA ablation except in the colon, where IL-10 further increased by twice ($p<0.05$). In the TNBS group, there was 4-12- and 4-7-fold increases in small intestinal IL-10 mRNA expression at 1 and 21 days after colitis induction, respectively (both $p<0.01$). This increase was not observed in rats pretreated with capsaicin. Capsaicin-treated and untreated rats had comparable visual ulcer scores after colitis induction.** ***Conclusion:*** **Inflammatory cytokines are produced *de novo* in distant intestinal segments in colitis. CSPA fibers play a key role in the upregulation of this synthesis.**

Keywords: inflammatory bowel disease, cytokines, TNF-α, IL-10, colitis, intestinal inflammation

Ulcerative colitis (UC) is generally confined to the colon with occasional extension into terminal ileum in what is known as 'backwash ileitis' [1]. However, many studies have shown some functional abnormalities in the small intestine of patients suffering from UC as well as in experimental animal models. These abnormalities manifested in histologically-intact small intestines [8, 9] as a decrease in intestinal fluid, D-xylose, amino acids, and fat absorption [2-6], as well as impaired gut motility [7]. The pathophysiology of this decrease however, has not been well elucidated. In fact, there are few reports of up-regulation of pro-inflammatory cytokines in macroscopically and microscopically unaffected mucosa of the colon of patients with UC and CD [10-13]. In rat models of acute and chronic colitis, increased levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 were demonstrated in distant and intact segments of the small intestine without any significant histological changes [14-16]. These studies revealed that this increase was not restricted to proinflammatory cytokines (TNF-α, IL-1β and IL-6), but also involved an upregulation of anti-inflammatory cytokines and mediators, such as IL-10 and NGF [17]. Both observations suggest a remote effect of colonic inflammation and its own autoregulatory mechanisms [17]. The question of how an inflammation or an ulcer in the colon may induce upregulation of cytokines at distant sites remains unanswered. Are these cytokines produced in the colon and then transported to the different parts of the small intestine by the blood stream (circulating cytokines) or are they secreted locally by the non-immune cells and/or residing

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immune cells through a neural reflex? Multiple studies have demonstrated cytokine-induced structural and functional derangements of the enteric nervous system (ENS) [18-23], but no studies have investigated the possible role for the ENS in the propagation of the inflammatory reaction from the colon to healthy proximal intestinal sites. Therefore, it is possible that the discrete cross-talk between the colon and the small intestine could be the result of a neuronal reflex, through the release of cytokines or some other neurohumoral mediators. The aim of this paper is to provide a detailed quantification of the transcription of inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokine gene expression in affected and unaffected segments of the gut, from 12 h up to three weeks in acute and chronic animal models of colitis. We also investigated the possible role of a major intestinal neural component, made of the capsaicin-sensitive primary afferents (CSPA), in the mediation of the expression of these cytokines at remote intestinal sites.

MATERIALS AND METHODS

Adult Sprague Dawley rats, weighing 200-250 g, were used for all experiments. They were housed under standard colony conditions (12 h light/dark cycle, 22 ± 2°C), with free access to a standard laboratory diet and water. They were starved for 24 h before the experiments, but had free access to tap water. All surgical procedures were performed under deep anesthesia with intraperitoneal pentobarbital (50 mg/kg). The study and the experimental protocols were approved by the Research Committee and the Institutional Animal Care and Use Committee at the American University of Beirut.

Induction of colitis

As previously described [24, 25], colitis was induced by transrectal instillation of 100 μL of 6% iodoacetamide (IA) or 250 μL of 50% ethanol containing 30 mg of 2, 4, 6-trinitrobenzene sulfonic-acid (TNBS), 7 cm proximal to the anal verge. Control rats received intracolonic injection of the same volume of the iodoacetamide (1% methylcellulose) or TNBS (50% alcohol) vehicles. Intracolonic injections were performed on awake rats gently restrained in a plastic cone (Decapicone, Braintree Inc., Braintree, MA, USA). The severity of colonic inflammation was assessed visually using a scale of 0-3, where a grade of zero was given to normal mucosa, and grades 1 to 3 for mucosal erosion, moderate lesion and deep lesion, respectively [25].

Ablation of capsaicin-sensitive primary afferents (CSPA)

Under halothane-induced anesthesia, rats received the first subcutaneous injection of capsaicin (25 mg/kg in 10% tween 80, 10% olive oil, 80% distilled water) followed by two additional injections (50 mg/kg) after 8 and 32 h. Rats were tested for successful ablation of CSPA 15 days later using the eye-wipe test. Colitis was then induced with IA and TNBS as described above.

Experimental groups

Data was obtained from four groups: the control group (n = 5-10), TNBS or IA-treated group (n = 5-10 rats each), capsaicin-treated group (n = 5), and TNBS or IA-treated group after successful ablation of CSPA (n = 5-8). IA-treated animals were sacrificed 12 and 48 h after colitis induction, and 48 h after CSPA ablation. Different groups of TNBS-treated animals were sacrificed 3 h and 1, 3, 7, or 21 days after colitis induction, and 1 and 21 days after CSPA ablation.

Determination of cytokine mRNA levels in intestinal mucosal scrapings

Under deep anesthesia, the abdominal cavity of the rats was opened along the midline. Segments of the mid-duodenum, mid-jejunum, mid-ileum and next to the ulcer site in the colon were removed, flushed with ice-cold normal saline, and cut along the anti-mesenteric border. Using glass slides, the mucosa was carefully scraped on ice, weighed and stored at -80°C.

RNA extraction

RNA was extracted using the guanidinium thiocyanate-phenol-chloroform extraction method. Each 50 mg of mucosal scrapings were homogenized for 20 to 30 s with 1 mL Trizol (Invitrogen) using a polytron homogenizer (Tissue Tearor). Homogenates were stored for 5 min at 4°C to permit the complete dissociation of the nucleoprotein complexes. Then 0.2 mL of chloroform per 1 mL trizol was added to trap the nucleic acids in the aqueous component, which was incubated on ice for 5 min after vigorous shaking, and then centrifuged at 4°C for 15 min at 11,000×g. The RNA present in the upper aqueous phase obtained after centrifugation was precipitated with 0.5 ml of isopropyl alcohol per 1 mL of RNA reagent used for the initial homogenization. Samples were incubated at 15 to 30°C for 10 min then centrifuged again at no more than 12,000×g for 10 min at 2 to 8°C. The RNA pellet obtained was washed with 75% ethanol, vortexed and then centrifuged for 5 min at 8600×g at 4°C .The extracted RNA was dissolved in RNAse-free water and stored at -80°C. The RNA obtained was quantified using the spectrophotometric quantification technique (Spectrophotometer Nanodrop ND-1000), using a 260/280 nm absorbance ratio method. A reverse transcriptase polymerase chain reaction (RT-PCR) was performed using specific primers for the target genes. Real-time PCR was performed on selected data as a confirmatory approach, and primarily on all the data obtained from the CSPA-ablated groups.

RT-PCR and gel electrophoresis

RT-PCR reactions were conducted in 200 μL, thin-walled PCR tubes, where 1 μL of the RNA of interest (0.5 μL for the β-actin gene) was added to 12.5 μL of a special mix (DNA polymerase, buffer, dNTP, and MgCl2), and 0.5 μL each of the sense primer, antisense primer and the reverse transcriptase enzyme. The final volume was adjusted to 25 μL by adding RNAse-free water. The positive control was created by omitting the reverse transcriptase in test samples to detect DNA contamination. Twenty μL of the amplified cDNA gene of interest (5 μL for the β-actin

gene) were loaded in 1.5% agarose gel made of 100 mL Tris Borate EDTA (108 g tris buffer, 55 g borate and 9.3 g of EDTA), 1.5 g agarose and 24 μL of 1 μg/μL ethidium bromide. The cDNA was run on the gel by applying a 100 mV voltage current. Amplicon size was determined using a Super ladder-low 100 bp ladder marker corresponding to 100-1000 bp. The intensity of each band was measured using the NIH image software. Correction for loading was achieved by subtracting for local background and normalization against corresponding β-actin mRNA levels. The same procedure was performed on control rats and the expression of genes in these animals was compared to treated ones.

Real-time PCR

Real-time PCR was conducted in two separate steps (QRT-PCR kit, ABgene, USA). The first step involved the reverse transcription of the m-RNA of interest to the corresponding c-DNA in 200 μL PCR tubes containing 1.33 μg of mRNA, 0.5 μL of reverse transcriptase, 1 μL of dNTP (deoxynucleotide phosphate), 0.5 μL of synthesis buffer and 0.5 μL of hexamer-oligo DT (deoxythymidylic acid) mix. The final volume was adjusted to 20 μL using RNAse-free water. The tubes were incubated at 42°C for 1 h and at 75°C for 10 min to initiate and terminate the reaction respectively. Amplification of the c-DNA obtained was performed using QPCR SYBR Green Mix (AB gene, USA). Each reaction consisted of 2.5 μL of c-DNA, 12.5 μL of SYBR green, 1 μl of each sense and antisense primer. The final volume was adjusted to 25 μL using RNAse-free water. The amplification was carried using the BioRad iCycler (BioRad, USA) for 45 cycles. The denaturation temperature was 95°C, the extension temperature was 72°C, and the annealing temperature varied depending on each cytokine's primer. A standard curve was obtained using a series of five samples with a 10-fold dilution. Data generated from the iCycler consisted of C_t (threshold cycle), which is the number of amplification cycles needed for the fluorescence to reach a designated background. Amplification specificity was checked using the melting curve technique. Q-Gene core module 1.2 software was used for data analysis, and normalization to the housekeeping gene (β-actin) was done.

Histology

Segments of the duodenum, jejunum, ileum and colon (proximal to the ulcer site) were isolated and processed for histology to determine if the change in tissue cytokine mRNA levels were accompanied by any signs of inflammation. The segments were removed and washed in ice-cold normal saline and fixed in 10% paraformaldehyde. They were then cut into 5-7 μm-thick sections and stained with hematoxylin and eosin (H&E) and periodic acid Schiff

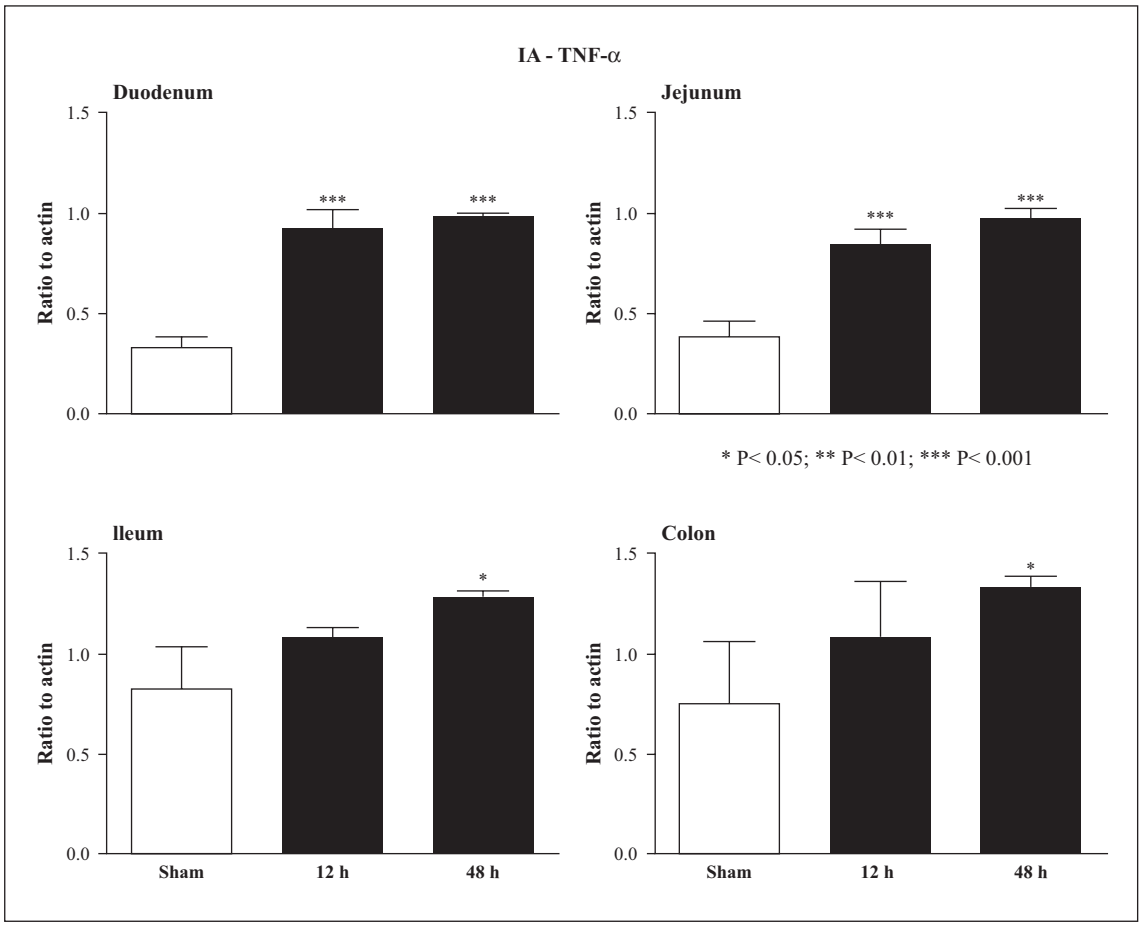

Figure 1
TNF-α mRNA expression using RT-PCR in mucosal homogenate obtained from various segments of the intestine in IA-induced colitis. Each bar represents mean ± SEM of values obtained from a different group of rats (n = 5-10 each) at the indicated time interval or control. The significance of the difference was obtained with reference to the values obtained from the control group.
IA, iodoacetamide

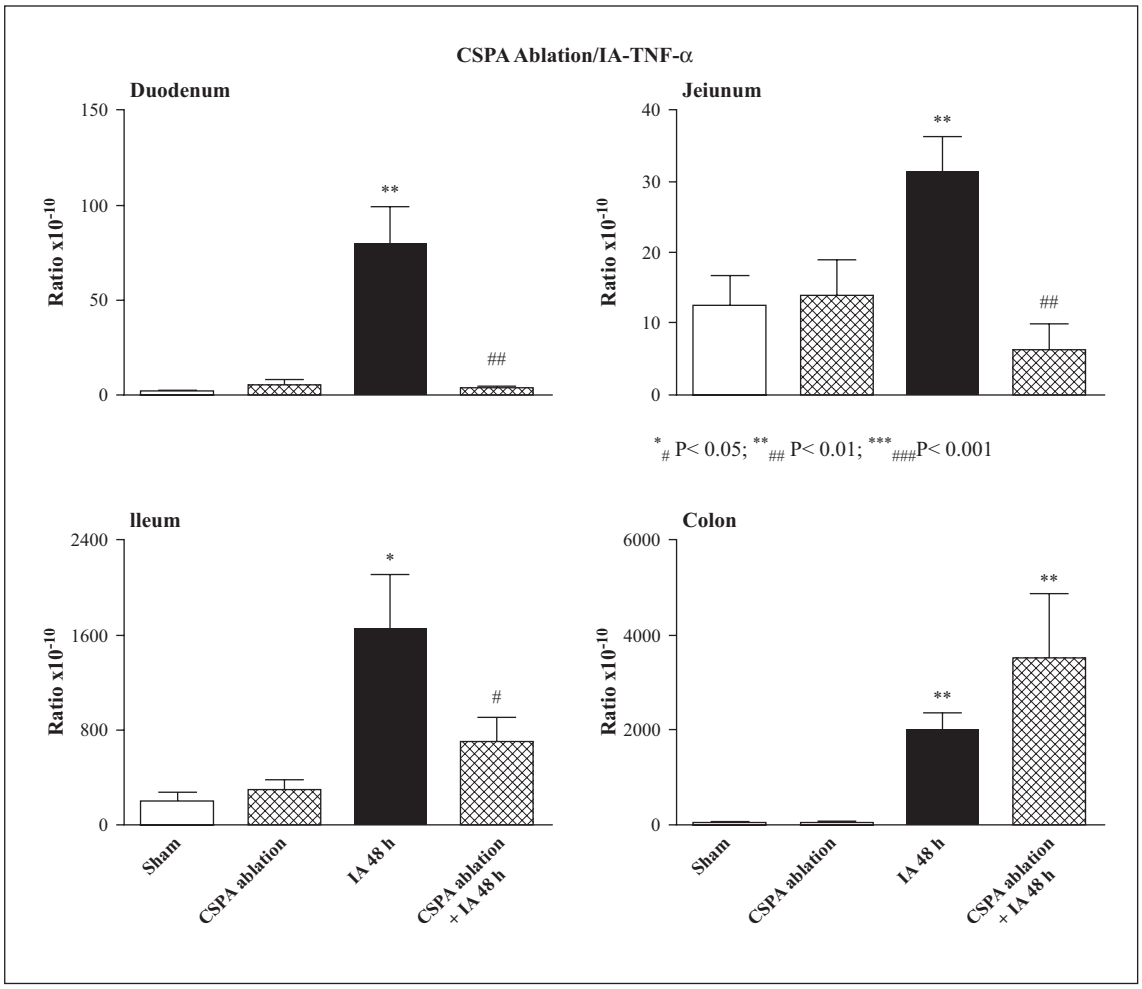

Figure 2

TNF-α mRNA expression using RT-PCR in mucosal homogenate obtained from various segments of the intestine in IA-induced colitis. Each bar represents mean ± SEM of values obtained from different animals within the control group (n = 5-10) and the treated groups (n = 5-10) at the indicated time interval, both before and after ablation of CSPA. The significance of the difference was obtained with reference to the values obtained from the control group before ablation of CSPA (*), and in reference to the corresponding experimental group within the same time line before ablation of CSPA (#).

CSPA, capsaicin-sensitive primary afferents.

(PAS) for routine light microscopy. Histological sections were examined by a pathologist who was blinded to the experimental protocols. The following criteria were taken into consideration for the assessment of each tissue sample: status of the intestinal lining, shape and alignment of enterocytes; shape and size of the villi; mast cells appearance and distribution; shape and content of goblet cells; changes affecting the basement membrane; intraepithelial lymphocytes, and any change in the structure and shape of the different intestinal layers. The presence and severity of any inflammatory cell infiltrates were also recorded.

Chemicals and reagents

Reagents used in this study included: sodium pentobarbital (AUB-MC pharmacy), Trizol (AB gene, UK.), iodoacetamide and 2, 4, 6, trinitrobenzene sulfonic-acid (Fluka, Switzerland), methyl-cellulose (Sigma Chemicals, St. Louis, MO, USA), chloroform (Amresco, Solon, Ohio), isopropanol (Amresco, Solon, Ohio), DEPC (diethyl pyrocarbonate) water, agarose (Amresco, Solon, Ohio), ethidium bromide (Amresco, Solon, Ohio), trishydroxymethylaminomethane (Biorad, USA), specific primers for TNF-α, IL-10 and β-actin, (AB gene, UK.), One Step RT-PCR kit (AB gene, UK.), two-step real-time PCR kit (Thermo, UK.).

Statistical analysis

The variations in mRNA levels for each cytokine of interest were obtained by averaging the values for each group of rats with colitis at the indicated time interval and comparing with the values obtained from the corresponding control group. Statistical analysis was performed using one-way ANOVA followed by *post hoc* tests. GraphPad Instat 3 and Prism 4-5 software were used for statistics and graphics, respectively (GraphPad Software Inc., San Diego, CA, USA). Data were expressed as means ± SEM for each group of rats, and significance was accepted at $p<0.05$.

RESULTS

Ulcer grading and small intestinal histology

In the IA model, the colonic ulcer grade was 3.0 at 12 and 48 h post-colitis induction, with no change after CSPA

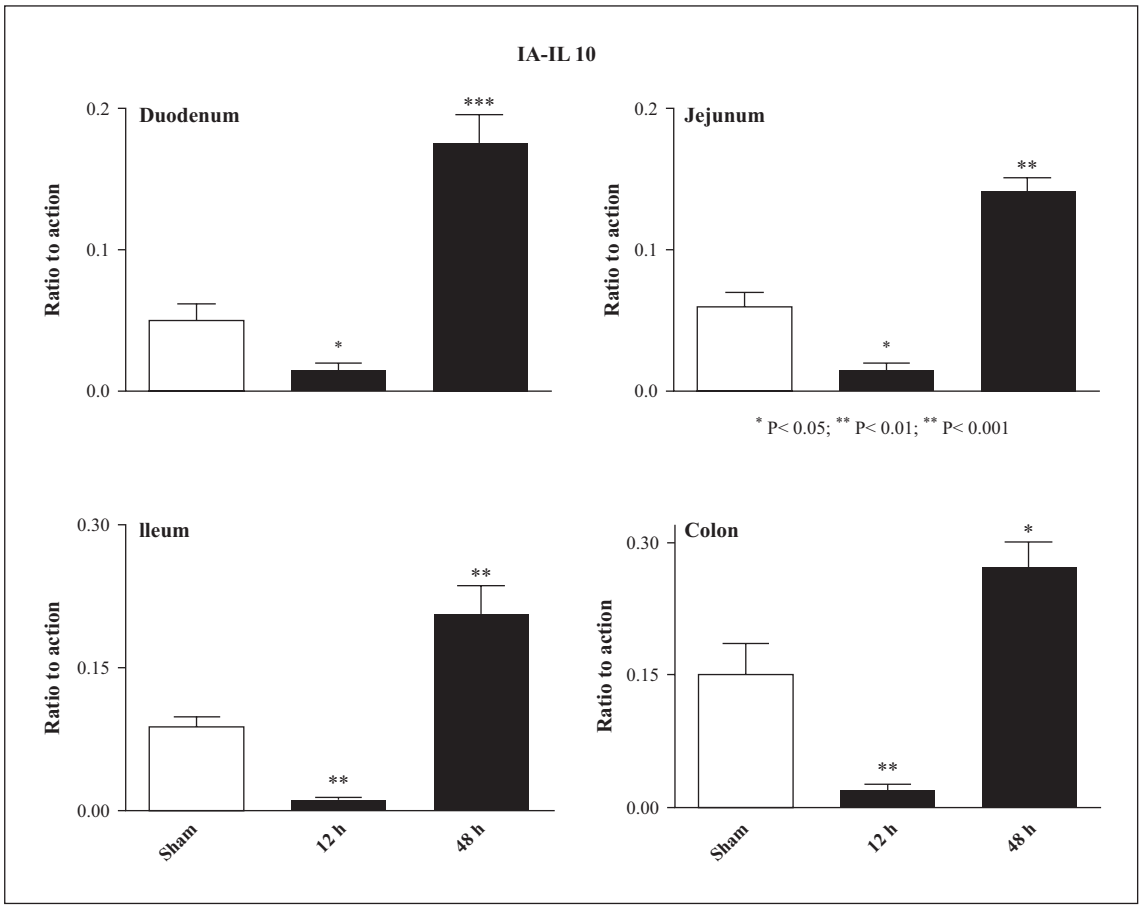

Figure 3
IL-10 mRNA expression using RT-PCR in mucosal homogenate obtained from various segments of the intestine in IA-induced colitis. Each bar represents mean ± SEM of values obtained from a different group of rats (n = 5-10 each) at the indicated time interval or control. The significance of the difference was obtained with reference to the values obtained from the control group.

ablation. In the TNBS model, the ulcer grade was zero at 3 h, 3.0 on days 1 and 3, 1.8 ± 0.58 on day 7, with complete healing on day 21. Similarly, CSPA ablation did not result in changes of the ulcer grade at 1 and 21 days.

Examination of sections sampled from various parts of the small intestine (including duodenum, jejunum and ileum) did not elicit overt signs of inflammation. The mucosal and submucosal components showed normal architecture in sections sampled from controls and rats treated with iodoacetamide or TNBS, with or without CSPA ablation.

Cytokine gene expression in IA-treated rats

Tumor necrosis factor alfa (TNF-α)

In the homogenate of the mucosal scrapings isolated from the duodenum of vehicle-treated rats, the level of TNF-α mRNA ratio-to-actin was 0.33 ± 0.05. Following treatment with IA, the TNF-α mRNA expression increased significantly to 0.93 ± 0.11 ($p<0.001$) and 0.98 ± 0.02 ($p<0.001$) at 12 and 48 h, respectively. A similar increase was observed in the jejunum (*figure 1*). However, an increase in TNF-α mRNA was observed in the ileum and colon at 48h only. The results were confirmed using real-time PCR where TNF-α mRNA expression in the duodenum increased from $1.74 \pm 0.29 \times 10^{-10}$ in control rats to $79.7 \pm 20 \times 10^{-10}$ ($p<0.01$) at 48h after IA instillation. The increase in TNF-α mRNA expression was totally inhibited in rats with CSPA ablation ($3.74 \pm 0.88 \times 10^{-10}$) ($p<0.01$). Comparable findings were observed in the jejunum and ileum. Interestingly, ablation of CSPA fibers elicited a further increase in colonic TNF-α expression (*figure 2*).

Interleukin 10 (IL-10)

In the homogenate of the muscosal scrapings isolated from the duodenum of vehicle-treated rats, the IL-10 mRNA-to-actin ratio was 0.05 ± 0.012. Twelve hours after the treatment with IA, IL-10 mRNA expression was suppressed, with a ratio to actin of 0.014 ± 0.006 ($p<0.05$). It then increased significantly above control values after 48h, with a ratio of 0.176 ± 0.02 ($p<0.001$). A similar pattern was observed in the remaining intestinal segments (*figure 3*). The results were confirmed using real-time PCR, where IL-10 mRNA expression in the duodenum increased from $1.88 \pm 0.58 \times 10^{-9}$ in control rats to $81.7 \pm 17.4 \times 10^{-9}$ ($p<0.01$) at 48h. The increase in the expression of IL-10 mRNA was significantly ameliorated in the capsaicin-treated rats ($8.2 \pm 1.7 \times 10^{-9}$) ($p<0.01$). Similar findings were observed in the jejunum. The increased mRNA expression in the ileum was not affected by desensitization to capsaicin. However, ablation of CSPA fibers resulted in a further increase in the expression of IL-10 in the colon (*figure 4*).

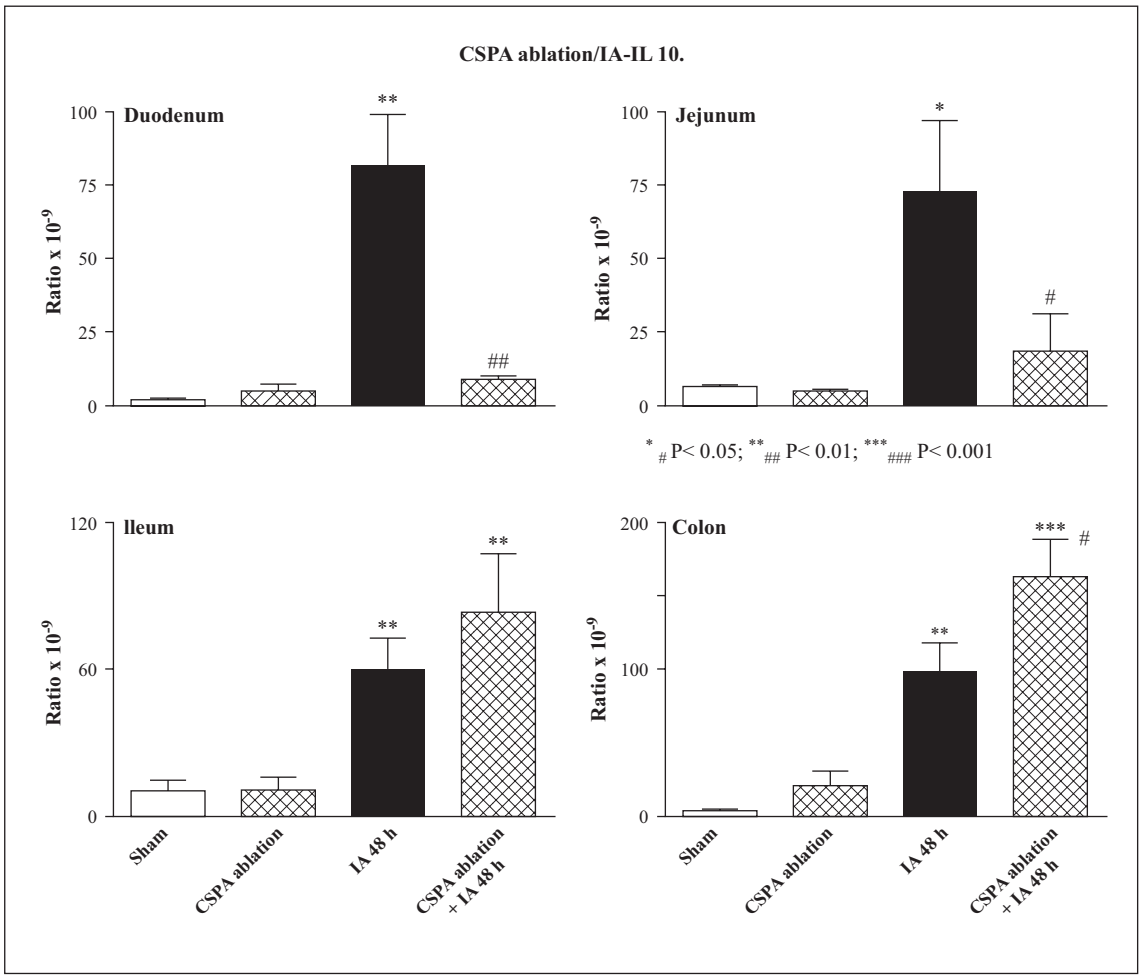

Figure 4

IL-10 mRNA expression using RT-PCR in mucosal homogenate obtained from various segments of the intestine in IA-induced colitis. Each bar represents mean ± SEM of values obtained from different animals within the control group (n = 5-10) and the treated groups (n = 5-10) at the indicated time interval, both before and after ablation of CSPA. The significance of the difference was obtained with reference to the values obtained from the control group before ablation of CSPA (*), and with reference to the corresponding experimental group within the same time line before ablation of CSPA (#).

Cytokine gene expression in TNBS-treated rats

Tumor necrosis factor alfa (TNF-α)

In the homogenate of the mucosal scrapings isolated from the duodenum of vehicle-treated rats, the TNF-α mRNA-to-actin ratio was 0.33 ± 0.05. Following treatment with TNBS, TNF-α mRNA expression increased significantly to 1.37 ± 0.3 ($p<0.003$) and 1.07 ± 0.3 ($p<0.02$) at 3 and 24 h, respectively. This was followed by a decrease in mRNA expression to 0.213 ± 0.02 at day 3 (similar to control values), then an increase at days 7 and 21 to 0.738 ± 0.08 ($p<0.01$) and 0.801 ± 0.07 ($p<0.01$), respectively. Similar findings were observed in the jejunum, ileum and colon (*figure 5*). The results were confirmed using real-time PCR where TNF-α mRNA expression in the duodenum increased from $2.14 \pm 0.5 \times 10^{-10}$ in control rats to $9.3 \pm 2.6 \times 10^{-10}$ ($p<0.05$) and $14.4 \pm 0.08 \times 10^{-10}$ ($p<0.001$) on days 1 and 21, respectively. Ablation of CSPA completely reversed the TNBS-induced up-regulation of TNF-α mRNA in the duodenum to $2.8 \pm 1.1 \times 10^{-10}$ ($p<0.05$ compared to TNBS-only) at day 1, but did not affect the expression at day 21 ($24.8 \pm 7.1 \times 10^{-10}$, $p<0.01$ compared to the control). The same confirmation of results and pattern of down-regulation after CSPA ablation was observed in the jejunum and ileum. As observed with the IA model, ablation of CSPA did not reduce TNF-α expression in the colon at any time interval, but actually increased it at day 21 (*figure 6*).

Interleukin 10 (IL-10)

In the homogenate of the mucosal scrapings isolated from the duodenum of vehicle-treated rats, the IL-10 mRNA-to-actin ratio was 0.057 ± 0.012. Following treatment with TNBS, IL-10mRNA expression increased significantly to 0.149 ± 0.04 ($p<0.01$) and 0.229 ± 0.036 ($p<0.001$) at 3 and 24h, respectively. This was followed by a gradual decrease at day 7, and a recovery to control levels at day 21 in all segments; in the colon, levels were comparable to those observed in control rats, except at 24 h (*figure 7*). The results were confirmed using real-time PCR where IL-10 mRNA expression in the duodenum increased from $1.89 \pm 0.59 \times 10^{-9}$ in control rats, to $22.5 \pm 5.4x \times 10^{-9}$ ($p<0.01$) at day 1, with the same results observed in the rest of the segments. Ablation of CSPA partially reversed the IL-10 expression at day 1 in the duodenum to $10.2 \pm 2.3 \times 10^{-9}$ ($p<0.01$ *versus* control, $p<0.05$ *versus* day 1), and completely in the jejunum ($p<0.01$), ileum ($p<0.01$), and colon ($p<0.05$). Ablation

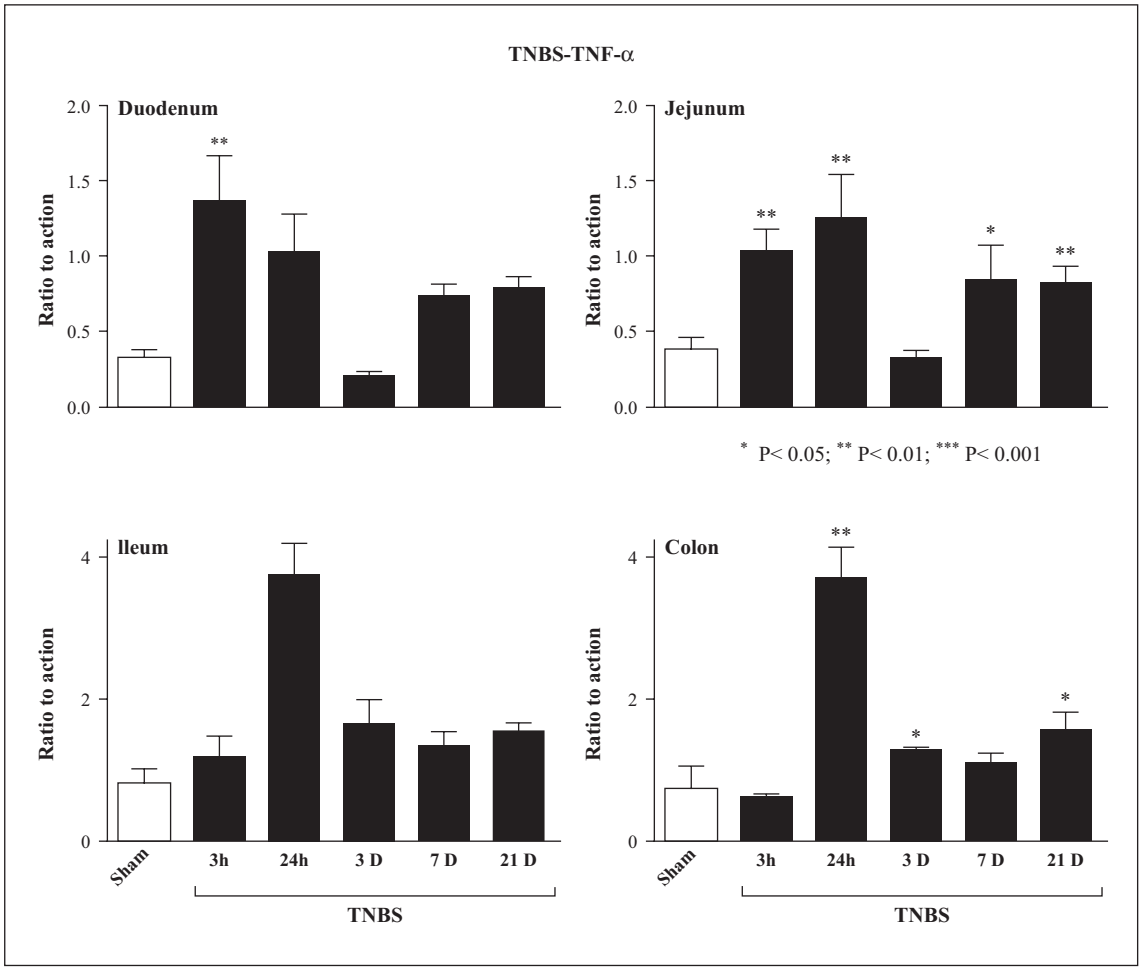

Figure 5

TNF-α mRNA expression using RT-PCR in mucosal homogenate obtained from various segments of the intestine in TNBS-induced colitis. Each bar represents mean ± SEM of values obtained from different animals within the control group (n = 5-10) and the treated group (n = 5-10) at various time intervals. The significance of the difference was obtained with reference to the values obtained from the control group. TNBS, trinitrobenzene sulfonic-acid.

of CSPA completely reversed the expression at day 21 in the duodenum ($p<0.01$), jejunum ($p<0.01$), and ileum ($p<0.05$) (*figure 8*).

DISCUSSION

In the present study, we have demonstrated that colonic inflammation in two rat models of colitis results in a significant *de novo* expression of pro and anti-inflammatory cytokines in distant and histologically normal intestinal segments. These data are in accordance with our previous findings of increased levels of these cytokines in remote intestinal sites [15, 17]. We also demonstrate a role for the intestinal innervation, specifically the CSPA fibers, in the spread of this colonic inflammation.

In both iodoacetamide- and TNBS-induced colitis models, the small intestine histology was normal, without any evidence of inflammation despite the increased expression of pro-inflammatory cytokines. We observed however, a low basal level of cytokine mRNA expression in different parts of the intestines under normal conditions, which goes hand-in-hand with our previous studies showing low basal protein levels of inflammatory cytokines in intestinal mucosal scrapings [15]. Other studies reported a colonic basal expression of TNF-α, IL-1β, and interleukin 6 (IL-6), and its increase in human IBD and experimental colitis [26-29]. This increase was observed in the inflamed mucosa of the colon, and may correlate with the severity of inflammation [26, 27, 30]. In humans with UC and Crohn's disease, an upregulation of pro-inflammatory cytokines has been described in both affected and unaffected areas of the colon, but markers of tissue damage and apoptosis (iNOS, MMP-3, GZNB) were elevated only in the affected segments [10-13]. In rat models of acute and chronic colitis, increased protein levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) as well as anti-inflammatory cytokines (IL-10 and NGF) were demonstrated in distant and intact segments of the small intestine with no significant histological changes [14-17]. These observations suggest a remote effect of colonic inflammation and its own independent autoregulatory mechanisms. It is still not clear how an ulcer or inflammation in the colon can upregulate cytokines at distant intestinal sites. These cytokines could either be transported from the site of inflammation (circulating cytokines), or alternatively secreted locally by non-immune cells and/or residing immune cells. In the present experiments, we found a significant increase in mRNA expression of these cytokines in various parts of the small intestine, demonstrating their *de novo* production in these remote intestinal areas in TNBS- and iodoacetamide-induced-colitis. We have previously demonstrated this

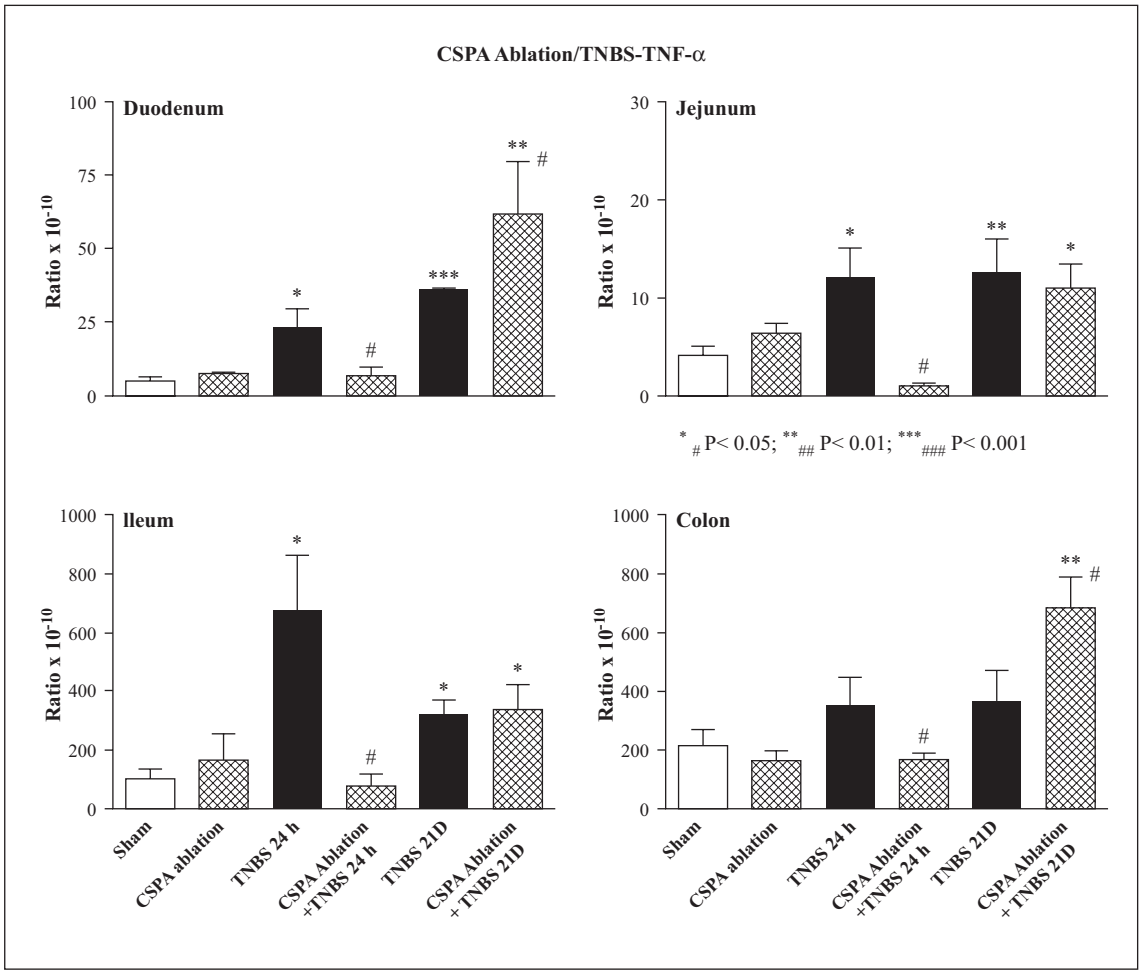

Figure 6

TNF-α mRNA expression using RT-PCR in mucosal homogenate obtained from various segments of the intestine in TNBS-induced colitis. Each bar represents mean ± SEM of values obtained from different animals within the control group (n = 5-10) and the treated groups (n = 5-10) at the indicated time interval, both before and after ablation of CSPA. The significance of the difference was made with reference to the values obtained from the control group before ablation of CSPA (*), and with reference to the corresponding experimental group within the same time line before ablation of CSPA (#).

phenomenon in a novel model of colitis induced by electrocautery [14, 16].

IL-10 acts as an immuno-modulator by inhibiting the production of pro- inflammatory cytokines [31] and up-regulating anti-inflammatory factors such as NGF [32] and IL-1 receptor antagonist [33]. The expression of IL-10 mRNA in the lamina propria and submucosa of patients with UC is inversely related to IL-1 [34]: IL-10 gene-knockout in mice results in spontaneous enterocolitis [35]. In our study, IL-10 mRNA expression was suppressed in affected and unaffected gut segments 12 h after colitis induction with IA. Comparable findings have been reported in biopsies obtained from endoscopically unaffected segments in patients with UC [13]. IL-10 expression in the colon was short-lived and decreased to control levels once ulcer healing had begun, unlike inflammatory cytokines where expression continued despite complete ulcer healing. Therefore, perpetuation of inflammation in colitis may be partially due to an imbalance between pro- and anti-inflammatory cytokines. Many studies suggest that several types of intestinal cells in intact intestinal segments can secrete both pro-inflammatory and anti-inflammatory cytokines [13, 34]. Given the absence of an overt inflammatory infiltrate in those segments, non-immune cells and/or residing immune cells are the probable source of cytokine secretion. In addition, epithelial cells in the unaffected segments were also reported to secrete TNF-α [13], IL-6 [13] and IL-10 [34].

In the TNBS model, there was a striking dissociation between the course of ulcer healing and the cytokine expression profile. While the colonic ulcer was still active three days after induction of colitis, TNF-α mRNA levels significantly decreased and reached control levels at day 7 in some intestinal segments, then increased again at day 21, despite the complete healing of the ulcer. However, IL-10 expression in the colon did not follow this biphasic pattern as it completely normalized when the ulcer healed. Previous studies have reported comparable findings. The number of IL-10-producing cells, the IL-10 tissue level [34] as well as the serum level of IL-10 [36], were found to correlate with the severity of inflammation in involved colonic segments of UC patients. The same was reported for IL-1 [10, 11, 29]. However, controversial evidence was reported about the correlation between increased TNF-α and IL-6 levels and the severity of inflammation [10, 11, 37, 38]. It is possible that once the inflammatory cascade is triggered, the presence of the active colonic ulcer may not be necessary to sustain it. This could be secondary to a permanent immune dysregulation induced by the colonic inflammation and may explain the chronicity of the disease and its remitting and relapsing nature. It is logical however, to expect a decreased

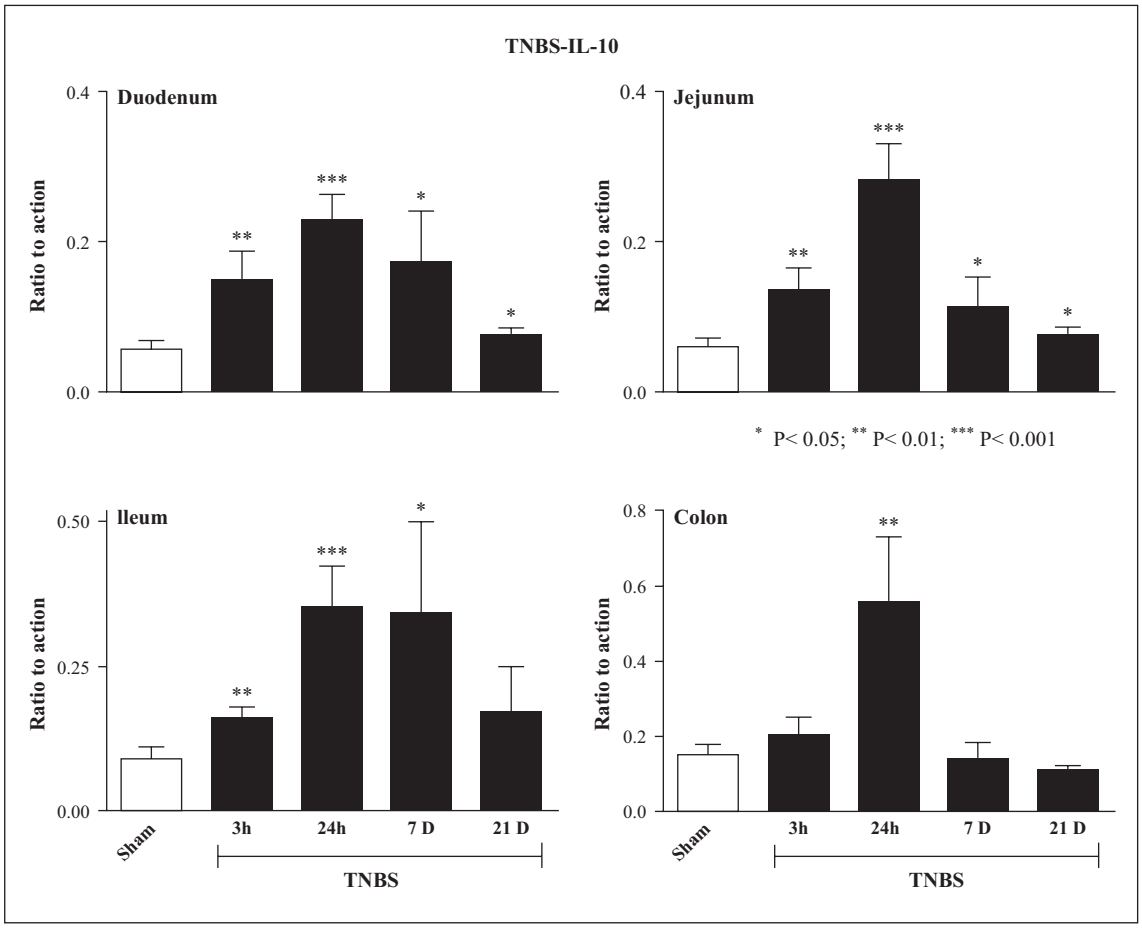

Figure 7

IL-10 mRNA expression using RT-PCR in mucosal homogenate obtained from various segments of the intestine in TNBS-induced colitis. Each bar represents mean ± SEM of values obtained from different animals within the control group (n = 5-10) and the treated group (n = 5-10) at various time intervals. The significance of the difference was obtained with reference to the values obtained from the control group.

expression of anti-inflammatory cytokines, such as IL-10, once tissue healing has occurred.

To test the hypothesis that this increase in cytokine production in remote intestinal areas is neurally mediated, we measured intestinal TNF-α and IL-10 expression in both colitis models in rats subjected to CSPA fiber ablation. In the IA model, CSPA fiber ablation completely inhibited the expression of TNF-α in duodenum and jejunum, although the level increased in the colon. IL-10 expression was significantly decreased in the duodenum and jejunum despite the fact that it increased in the ileum and colon. In TNBS-induced colitis, CSPA ablation had an inhibitory effect on TNF-α expression in the early acute phase, but not in the late or chronic phase. IL-10 expression was inhibited in both acute and chronic phases.

Early clinical observations pointed out the fact that the spreading of the effect of colitis to other inflamed and non-inflamed areas might involve the GI innervation that can play a dual role as a modulator or as a carrier of inflammation [22, 39-41].

Several studies have shown that the acute inflammatory response to TNBS can alter the function of the ENS at both inflamed and non-inflamed sites in the intestine [42, 43], and that exogenous application of IL-1β in control tissue can mimic this change in neural function [44]. In addition, cytokines such as IL-1 and IL-3, present in the inflamed intestine can directly affect intestinal neural function by altering neurotransmitter release and acutely affecting the ENS [23], or by inducing neuronal death followed by abnormal sprouting of axons from the remaining neurons [45]. Blandizzi *et al*. [18] presented convincing evidence that experimental colitis in rats leads to distinct changes in the neural circuitry of non-inflamed regions of the bowel, and suggests that this may underlie the changes in small bowel motility that accompany colitis. On the other hand, the vanilloid receptors TRPV1, located on the CSPA fibers, have been shown to mediate the endogenous mechanisms protecting against induced colonic ulcer [46, 47]. The protective effect of the extrinsic afferents is further enhanced by the anti-inflammatory role played by the parasympathetic efferents to the GI tract [48, 49].

There is evidence of a complex neuro-immune interaction that underlies the phenomenon of cross-talk between different parts of the intestine. Enteric nerve endings immuno-reactive to vasoactive inflammatory peptide (VIP), somatostatin (SOM) and substance P (SP) lie in proximity to immune cells inside Peyer's patches, the lamina propria and the submucosa [50-55]. Direct membrane-to-membrane contact has been described between mast cells and SP/CGRP-IR axons in human and rat intestine [56-58]. In TNBS-induced colitis, a close spatial relation has been observed between SP/VIP-immunoreactive (IR) nerve fibers and lymphoid cells in the mucosal and submucosal layers [50]. Interestingly, human and rodent immune cells express SOM and VIP receptors [56, 59], as well as the high affinity and

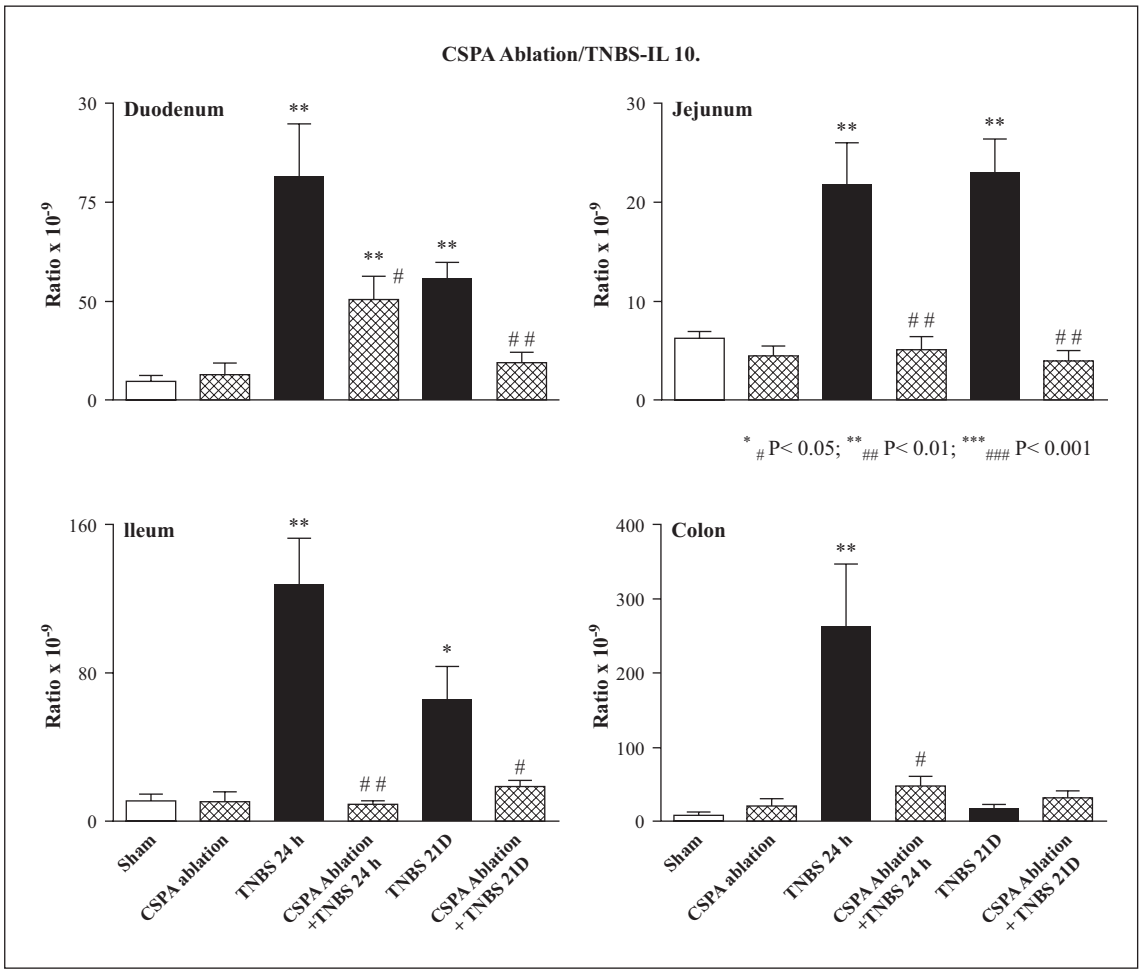

Figure 8

IL-10 mRNA expression using RT-PCR in mucosal homogenate obtained from various segments of the intestine in TNBS-induced colitis. Each bar represents mean ± SEM of values obtained from different animals within the control group (n = 5-10) and the treated groups (n = 5-10) at the indicated time interval, both before and after ablation of CSPA. The significance of the difference was obtained with reference to the values obtained from the control group before ablation of CSPA (*), and with reference to the corresponding experimental group within the same time line before ablation of CSPA (#).

specificity SP receptor (NK-1R). The latter is also expressed on endothelial and epithelial cells [60-67]. The expression of this receptor is increased in patients with UC and CD, mostly on the epithelial cells lining the mucosal surface and crypts, but also on endothelial cells of capillaries and venules [67] in both affected and unaffected intestinal segments. Once released from their nerve terminals, neuropeptides (namely SP) stimulate cytokine secretion by immune [60, 68-72] and epithelial cells [60]. Pro-inflammatory cytokines reciprocally stimulate further SP and CGRP release from neurons [73] and expression of NK-1R in human macrophages. Capsaicin treatment in rats permanently depletes SP and SOM in sensory nerves and in the dorsal half of the spinal cord [74, 75] which would interrupt this neuro-immune circuit and explain the reversal of cytokine release in some gut segments after CSPA ablation. The neuro-immune interaction is not unidirectional as SP, VIP and SOM have been isolated from immune cells [55, 60, 63, 76, 77], while the NK-1R receptor is also localized on the ENS nerve terminals [60, 78, 79]. The presence of neuropeptides and their respective receptors on both the ENS fibers and the gut immune cells points towards a bidirectional communication between the two systems.

We believe that colitis-induced mucosal damage activates sensory afferent fibers leading to the distant release of neuropeptides, which, in turn, stimulate the synthesis and release [15] of cytokines from local immune and non-immune cells of the healthy gut. Inflammatory cytokines further potentiate this reaction through a reciprocal mechanism. It appears, however, that this hypothesis applies only during the early stages of colitis (acute and early chronic), and is more pronounced the further the distance from the colonic ulcer, as some cytokines in the ileum and colon were not suppressed at any time interval, despite CSPA ablation: most cytokines in various gut segments peaked again at 21 days when the colonic ulcer has completely healed. The first possible explanation for this "double peak" pattern is that the distant immune response, once triggered, would follow a different course in relation to the colonic ulcer. Another, more likely explanation is the early loss of SP caused by destruction of SP-IR nerve fibers and/or depletion due to excessive release from nerve terminals [80]. This leads to a temporary interruption of the neuro-immune circuit and the observed decrease in distant cytokine production. The later reconnection of the neuro-immune circuit will resume the stimulus for distant cytokine production.

In conclusion, we report that induction of colonic ulcers results in *de novo* synthesis of inflammatory and anti-inflammatory cytokines in remote, unaffected areas of the gastrointestinal tract. We also point toward the potential

role of the enteric nervous system in propagating some aspects of the inflammatory reaction. The loss of distant cytokine expression after CSPA ablation proves the underlying role of the ENS in regulating the immune functions in the gastrointestinal tract, and emphasizes its complex neuro-immune interactions.

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