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Activation of μ Opioid Receptors Modulates Inflammation in Acute Experimental Colitis

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Abstract

Background—μ opioid receptors (μORs) are expressed by neurons and inflammatory cells and mediate immune response. We tested whether activation of peripheral μORs ameliorates the acute and delayed phase of colitis.

Methods—C57BL/6J mice were treated with 3% dextran sodium sulfate in water, 5 days (DSS) with or without the peripherally-acting μOR agonist, [D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin (DAMGO) or with DAMGO+μOR antagonist at day 2–5, then euthanized. Other mice received DSS followed by water for 4 weeks, or DSS with DAMGO starting at day 2 of DSS for 2 or 3 weeks followed by water, then euthanized at 4 weeks. Disease activity index (DAI), histological damage, and myeloperoxidase assay (MPO), as index of neutrophil infiltration, were evaluated. Cytokines and μOR mRNAs were measured with RT-PCR, and nuclear factor-kB (NF-kB), the antiapoptotic factor Bcl-xL, and caspase 3 and 7 with Western blot.

Key Results—DSS induced acute colitis with elevated DAI, tissue damage, apoptosis and increased MPO, cytokines, μOR mRNA and NF-kB. DAMGO significantly reduced DAI, inflammatory indexes, cytokines, and caspases, and NF-kB, and upregulated Bcl-xL, effects

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DISCLOSURE

The authors have no competing interests

LA, JH, CD, IJ, GV, & FS performed the research; LA, CD, RDG & CS designed the research study and analyzed the data; LA, CD, RDG, NCB & CS wrote the article.

prevented by μOR antagonist. In DSS mice plus 4 weeks of water, DAI, NF-kB and μOR were normal, whereas MPO, histological damage and cytokines were still elevated; DAMGO did not reduce inflammation, and did not upregulate Bcl-xL.

Conclusions & Inferences—μOR activation ameliorated the acute but not the delayed phase of DSS colitis by reducing cytokines, likely through activation of the antiapoptotic factor, Bcl-xL, and suppression of NF- kB, a potentiator of inflammation.

Keywords

anti-apoptotic factor; cytokines; inflammatory indexes; transcriptional nuclear factor kB

μ opioid receptors (μORs) are G-protein coupled receptors (GPCRs) that are activated by endogenous opioid peptides and structurally distinct alkaloids, drugs commonly used for pain control [1, 2]. μORs are abundantly expressed in the central nervous system [3, 4], where they mediate analgesia and addiction [5, 6], and the enteric nervous system [7–9], where they mediate gastrointestinal (GI) motility, secretion and electrolyte and fluid transport [10–12]. μORs and their ligands are also localized to different types of immune cells such as neutrophils, macrophages, and T and B cells throughout the body, including the GI tract [9, 13–15]. Furthermore, immune cells secrete endogenous opioids and mediate inflammatory responses by direct interaction with opioid receptors [16–18]. The widespread distribution of μORs parallels the broad spectrum of biological functions mediated by these receptors when activated by endogenous opioid peptides and opioid drugs in the CNS and periphery, including immune responses [5, 6, 11, 12, 15, 17, 19].

There is increasing evidence that μORs play a protective role in different inflammatory conditions of the digestive system. Upregulation of μOR mRNA has been reported in different animal models of intestinal inflammation [20, 21] and in patients with inflammatory bowel disease (IBD) [22], whereas μOR mRNA expression was impaired in chronic hepatitis [23]. Furthermore, activation of μORs with selective agonists has been shown to protect from intestinal inflammation induced by ischemia/reperfusion[24], prevent acute hepatitis [23] and ameliorate acute experimental colitis induced by intrarectal 2,4,6-trinitrobenzebesulfonic acid (TNBS) [20].

The above findings provided the background for the current study, which was designed to test the effect of μOR activation on colitis induced by dextran sodium sulfate (DSS), a well-established, chemically induced model of colitis that is strongly dependent on innate immune response [25] during the acute and delayed phase of inflammation. We first determined whether μOR expression correlates with clinical symptoms and local and systemic inflammatory indexes, including inflammatory cell infiltration, and cytokines levels. To determine the mechanisms underlying the effect of μOR activation on inflammation, in addition to the inflammatory indexes, we evaluated the tissue expression of Bcl-xL, an antiapoptotic member of the Bcl-2 family of apoptosis regulator proteins, which protects from injury-induced cell death [26–28], apoptosis and the transcription nuclear factor-kB (NF-kB), which potentiates inflammatory and immune responses by inducing cytokines production [29, 30]. This study suggests that activation of peripheral μORs ameliorates inflammation in acute experimental colitis by reducing cytokine production,

cellular infiltration, tissue damage and apoptosis through the suppression of NF-kB and the upregulation of Bcl-xL, but does not have any detectable effect during the delayed phase of inflammation.

MATERIALS AND METHODS

Experiments were performed on adult C57BL/6J mice (20–25 g; Charles River Laboratory International, Inc, Hollister, CA) housed in rooms with controlled temperature and light-dark cycles. Mice had unrestricted access to standard mouse chow and tap water and acclimated for one week before experimentation. Animal care and procedures were in accordance with the National Institutes of Health recommendations for the humane use of animals. Experimental procedures were reviewed and approved by the Animal Research Committee of the University of California, Los Angeles. All efforts were made to minimize the number of animals used and their suffering. Animals were closely monitored by the Veterinarian and the animal welfare was assessed to assure they did not develop signs of discomfort, which would have warranted interruption of treatment and/or euthanasia.

Induction of Colitis

In one set of mice (total 56), acute colitis was induced by administering 3% (w/v) DSS (40,000 – 50,000; Affymetrix, Inc., USA) in drinking water *ad libitum* for five days. Control mice (n=14) received only drinking water. Initial studies compared the effect of different concentrations of the selective μOR agonist, [D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin (DAMGO; at 0.01, 0.02, 0.04 and 0.08 mg kg^{-1} s.c. a day) starting 2 days after the beginning of DSS administration to the end of treatment. The dose of 0.02 mg kg^{-1} × day of DAMGO was selected for the subsequent experiments, because it was the lowest dose that induced the maximum effect on the reduction of the disease activity index and the myeloperoxidase activity (see below, Results). To determine that DAMGO effects were receptor-mediated, mice treated with DSS received the highly selective μOR antagonist, [H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2] (CTAP, 0.2, 2.0 and 4.0 mg kg^{-1} × day) [31] in addition to DAMGO. All CTAP doses induced significant inhibition of DAMGO-induced effects, but the 2.0 mg had less variability therefore it was selected for subsequent studies. The effects of systemically administered DAMGO are due to the activation of peripheral μORs, since DAMGO does not readily pass the blood-brain barrier [32]. CTAP passes the blood brain barrier [33], but it does not block the effect of systemic DAMGO on μORs when it is administered intracerebroventricularly [32]. To determine the role of Bcl-xL on DAMGO effect in experimental colitis, a set of mice that received DSS in drinking water was treated with ABT 737 (40 mg kg^{-1} daily; Selleck Chemicals, Houston, TX) [34], a selective inhibitor of the Bcl-2 family, including Bcl-xL, in the presence or absence of DAMGO.

Another set of mice (total 30) received a single cycle of DSS treatment (3% in drinking water *ad libitum* for five days as above) followed by 4 weeks of drinking water only, or a single cycle of 3% DSS with DAMGO, 0.02 mg kg^{-1} a day starting at day 2 of DSS administration and continuing after the end of DSS for two weeks or three weeks and then

water to the end of the 4 weeks. With this paradigm, C57BL/6J mice euthanized 4 weeks following the end of a single cycle of DSS showed active colitis [35].

Mice were weighed daily and visually inspected for diarrhea and rectal bleeding. Mice were euthanized at the end of each experimental procedure (either at the end of 5 days DSS treatment or at 4 weeks following the end of DSS treatment) with an overdose of Isoflurane for tissue collection. Colon from the colo-cecal junction to the anus was removed from each animal and flushed with cold phosphate-buffered saline. Tissue obtained from each colon was processed for histology, Western blot, myeloperoxidase activity and quantitative RT-PCR (qRT-PCR).

Disease Activity Index

Clinical assessment of inflammation included daily monitoring of body weight, stool consistency, and fecal blood. The disease activity index (DAI) was calculated according to a modified protocol [36] by grading loss of weight on a scale of 0 to 4 (0= normal; 1= 0–5%; 2= 5–10%; 3=10–20%; 4 >20%), stool consistency on a scale of 0 to 2 (0= normal; 1= loose stools; 2= diarrhea), and presence of fecal blood on a scale of 0 to 2 (0=normal; 1= bleeding; 2= severe bleeding). The final DAI value for each animal was expressed as the average of the combined scores.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an index of tissue neutrophil infiltration, was measured in the colon as previously described [24]. Briefly, a portion of colon was homogenized in 1:20 (w/v) of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma-Aldrich, ST. Louis, MO) on ice using a Polytron homogenizer. The homogenate was centrifuged at 14,000 rpm for 15 min. Supernatant at 4°C was added to o-dianisidine hydrochloride solution (Sigma-Aldrich, ST. Louis, MO) containing 0.0005% hydrogen peroxide, and the change in absorbance at 460 nm was measured. One unit of MPO activity was defined as the amount that degraded 1 μmol peroxidase per minute at 25°C. The results were expressed as U mg^{-1} of tissue. The rate of change in absorbance was measured with a spectrophotometer at 470 nm (Bio-Tek, Winooski, VT).

Histological damage

For the evaluation of the microscopic histological damage, colon specimens were fixed in 10% formalin/phosphate buffer saline overnight, embedded in paraffin and sectioned. Tissue sections (4 μm) were stained with hematoxylin and eosin. Microscopic histological damage score was evaluated by a person unaware of the treatments and was based on a semiquantitative scoring system [24, 37] in which the following features were graded: damage of epithelium (0 = morphologically normal; 1 = zonal destruction of the epithelial surface; 2 = diffuse epithelial destruction and/or mucosal ulcerations involving submucosa; 3 = severe epithelial destruction), inflammatory cell infiltration (0 = absence of infiltrate or fewer than five cells; 1 = mild infiltration to the lamina propria; 2 = moderate infiltration to the muscularis mucosae; 3 = high infiltration to the muscularis mucosae; 4 = severe infiltration involving the submucosa), separation of muscle layer and muscularis mucosae (0 = normal; 1 = moderate; 2 = severe), goblet cells depletion (0 = no depletion; 1 = presence

of non-organized goblet cells; 2 = presence of 1 to 3 areas without goblet cells; 3 = more than 3 areas without goblet cells; 4 = complete depletion of goblet cells). The total score of individual mice was expressed as the average of the sum of the different histological subscores.

Cytokines and μOR mRNA Expression in the Colon: Real-Time Quantitative RT-PCR

We selected RT-qPCR as a measure of cytokine expression to compare the effect of μOR activation on DSS-induced colitis to the effects on TNBS-induced colitis [20] and intestinal ischemia/reperfusion [24]. Samples of colon were homogenized and total RNA was extracted using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA). The purity and amount of the purified RNA was determined by spectrophotometry (Nanodrop 1000; Thermo Scientific, Waltham, MA). The NanoDrop measured the quantity and quality of a 1uL sample of extracted RNA on the basis of the optical density 260 and optical density ratio 260/280, respectively. Only the RNA with an optical density 260/280 ratio greater than 1.8 was used for RT-PCR gene expression analysis. The integrity of the total RNA was assessed by electrophoresis on a denaturing agarose gel stained with GelRed™, in which the 18S and 28S ribosomal RNA bands were inspected. The expression of μOR and cytokines, tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, IL-6 and IL-10 mRNA was assessed by qRT-PCR using TaqMan Gene Expression Master Mix and Pre-Developed TaqMan Assay (Applied Biosystems, Foster City, CA) specific to mouse μOR (Mm01188089), TNF-α (Mm99999068_m1), IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), IL-10 (Mm01288386_m1) and the housekeeping gene β-actin (Mm1205647_g1). The data were collected using a Stratagene Mx 3000p machine. Expression of β-actin was measured in parallel for every sample, and the data obtained for μOR, TNF-α, IL-1β, IL-6 and IL-10 were normalized to those of the β-actin. Relative quantities (RQ) of mRNA were analyzed using the comparative threshold cycle (Ct) method [38, 39].

Western Blotting analysis of Bcl-xL, NF-kB, caspase 3 and caspase 7

Whole protein extracts were prepared from mouse frozen colon samples. Tissues were homogenized using PowerGen homogenizer (Fisher Scientific, Torrance, CA) in 1 mL of lysis buffer. After incubation on ice for 30 minutes, protein extracts were centrifuged for 20 minutes at 4°C; supernatant fractions were collected and their protein contents were quantified using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Samples were diluted (v/v) in Laemmli sample buffer (pH 6.8) and boiled 10 minutes before loading onto gel. Homogenates (45 μg) were fractionated using SDS-10% polyacrylamide electrophoresis gel (65V, overnight) and pre-stained marker proteins were used as molecular mass standards (Bio-Rad Laboratories, Hercules, CA). Separated proteins were transferred electrophoretically to PVDF Immobilon-FL membranes (Millipore, Temecula, CA). Membranes were blocked 1 hour at room temperature in LI-COR Blocking buffer (LI-COR Biosciences, Lincoln, NE) and incubated at 4°C overnight with a rabbit polyclonal anti-Bcl-xL (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA), NF-kB antiserum (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti cleaved-caspase-3 or cleaved-caspase 7 (1:1000; Cell Signaling, Danvers, MA) or a mouse anti GAPDH antibody (Cell Signaling, Danvers, MA) that served as housekeeping protein. Cleaved caspase 3 and 7 are the

activated caspase forms leading to apoptosis [40]. Blots were washed and incubated 2 hours at room temperature with an infrared fluorescent secondary antibody IRDye 680 goat anti-rabbit or IRDye 800 goat anti-mouse (1:10,000). Images were collected using the LI-COR Odyssey Infrared Imaging System and intensities were quantified using the Odyssey Application Software Version 3.0. Bcl-xL and NF-kB protein levels were normalized to GAPDH levels.

Statistical analysis

Data were expressed as mean ± SE. Comparison among groups was made by using one-way ANOVA followed by the post hoc Bonferroni test, which corrects for any variance that compares means from unequal sample sizes. Student-t test was used when comparing only two groups. Differences were considered significant at $p<0.05$. Analysis was performed in Prism 5 (GraphPad Software, San Diego, CA).

RESULTS

Induction of colitis and disease activity index (DAI)

Mice treated with 3% DSS for 5 days had a significant increase of DAI ($p<0.01$) compared to control animals that received tap water, consistent with the development of acute colitis (Fig. 1). Treatment with DAMGO at 0.02 mg kg^{-1} × day induced a significant reduction of DAI ($p<0.05$). DAMGO at the lowest (0.01 mg kg^{-1}) and higher doses (0.04 or 0.08 mg kg^{-1}) did not affect DAI (Fig. 1). Similar data were obtained with MPO (see below), which is consistent with previous studies using different inflammation models [20, 24]. Therefore, we used 0.02 mg kg^{-1} × day of DAMGO for all subsequent experiments. In order to demonstrate that the effect of DAMGO was mediated by activation of μOR, we used increasing concentrations of the antagonist CTAP (0.2, 2 or 4 mg kg^{-1} × day), which was administered at the same time as DAMGO (0.02 mg kg^{-1}). These experiments showed that all the doses of CTAP significantly reversed the effect of DAMGO on DAI ($p<0.05$–0.01), but CTAP at the 2 mg kg^{-1} had the lowest variability compared to other doses (Fig. 1).

In the group of mice treated with 1 cycle of DSS and followed for 4 weeks from the end of the DSS treatment before euthanasia, there was a progressive and significant decrease in body weight starting at day 6 to day 19 ($p<0.05$–0.001). Body weight slowly increased back to initial values at day 21 and continued to increase up to day 34 (Fig. 2A). In mice that received 0.02 mg kg^{-1} × day of DAMGO during DSS administration followed by 0.02 mg kg^{-1} × day of DAMGO for 2 weeks and then water for 2 more weeks, the loss in body weight between days 8–10 was significantly less compared to mice that did not receive DAMGO (Fig. 2A). Mice also showed a progressive and significant increase of DAI from day 3 to day 19 ($p<0.001$) (Fig. 2B), which was significantly reduced by treatment with DAMGO between days 3–6 (Fig. 2B). The results in animals that received DAMGO for 3 weeks followed by 1 week of water were completely superimposable with the ones obtained with 2 weeks of DAMGO following DSS treatment shown here.

MPO Activity

Mice treated with 3% DSS showed a significant increase in intestinal MPO activity ($p<0.001$) compared to control (Fig. 3). DAMGO at 0.02 mg kg^{-1} × day significantly reduced MPO activity ($p<0.01$) (Fig. 3), and this effect was reversed by the μOR antagonist CTAP. DAMGO at 0.01 mg kg^{-1} × day did not modify MPO values, whereas higher concentrations of DAMGO (0.04 and 0.08 mg kg^{-1}) reduced the MPO response, but not significantly (Fig. 3). All concentrations of CTAP reversed the DAMGO-effect on MPO ($p<0.05$–$p<0.001$) (Fig. 3). CTAP (2 mg/Kg) alone did not significantly affect the MPO activity in mice with DSS-induced acute colitis (CTAP+DSS 2.80±0.29 vs. DSS 2.50±0.17, n=4 each group).

In mice euthanized 4 weeks following DSS treatment, MPO activity was still significantly increased compared to control ($p<0.001$), though significantly less compared to the acute colitis ($p<0.01$) (Fig. 3). Treatment with DAMGO for 2 or 3 weeks followed by water did not have any effect on MPO activity (Fig. 3) compared to mice that only received water for 4 weeks following the DSS cycle.

Intestinal Histology

There was significant tissue damage in mice treated with 3% DSS for 5 days ($p<0.001$ vs. controls), which included pronounced infiltration of inflammatory cells within lamina propria and submucosa, severe edema and lifting of the epithelial layer from the lamina propria (Fig. 4), crypt abscesses, pronounced depletion of goblet cells and muscle thickening compared to control (Fig. 4). Treatment with DAMGO significantly reduced DSS-induced histological damage ($p<0.001$ vs. DSS; Fig. 4). This effect was completely reversed by the co-administration of the μOR antagonist peptide, CTAP ($p<0.05$ vs. DAMGO treated; Fig. 4).

Tissue damage was still significant in mice at 4 weeks following DSS treatment compared to controls ($p<0.001$), but it was reduced compared to the tissue damage observed in acute colitis ($p<0.01$). However, tissue damage in these animals was not affected by either of the DAMGO treatment regimens (Fig. 4).

Cytokine mRNA Expression

The levels of TNF-α, IL-1β and IL-6 mRNA quantified by qRT-PCR were markedly and significantly increased in the colon of mice with acute colitis compared to control ($p<0.001$) (Fig. 5). Treatment with DAMGO significantly reduced the levels of TNF-α, IL-1β and IL-6 mRNA compared to animals treated with DSS only (Fig. 5) ($p<0.001$). The levels of IL-10 were very low overall, compared to the other cytokines. There was only a trend, but not significant increase in the expression of IL-10 mRNA in acute DSS colitis (Fig. 5), which was significantly reduced by DAMGO treatment ($p<0.05$). DAMGO effects on cytokine expression were abolished by CTAP (Fig. 5).

Four weeks after DSS treatment, there was still a significant increase of TNF-α and IL-6 mRNA levels compared to control ($p<0.05$–$p<0.01$), however the levels of these cytokines were significantly lower compared to the levels in acute colitis ($p<0.01$-$p<0.001$). On the

other hand, the levels of IL-1β and IL-10 were not increased in these mice compared to controls. Treatment with DAMGO for either 2 or 3 weeks after the end of DSS treatment did not affect any of these cytokines levels (Fig. 5).

μOR mRNA expression in DSS-induced colitis

Acute colitis induced by 3% DSS for 5 days triggered a significant increase in the levels of μOR mRNA compared to control mice (1.71 ± 0.2 vs. 1.07 ± 0.11, n=8 in each group) ($p<0.001$). μOR mRNA levels 4 weeks following DSS treatment (n=5) were significantly lower than in acute colitis (0.42 ± 0.03 vs. 1.71 ± 0.2; $p<0.001$), and not significantly different from controls.

Expression of Bcl-xL and NF-kB in DSS-induced colitis

Mice treated with 3% DSS and injected with DAMGO presented a significant increase in Bcl-xL expression ($p<0.001$) compared to control mice, effect that was abolished by CTAP administration ($p<0.001$) (Fig. 6). CTAP alone did not affect the expression of Bcl-xL in DSS acute colitis (86.52±9.1% in DSS+CTSAP vs. 103.7±10.14% in DSS alone). By contrast, in mice that were followed for 4 weeks after DSS treatment, there was no increase in Bcl-xL levels in response to DAMGO (Fig. 6). DAMGO had no effect on the expression of Bcl-xL in normal mice (data not shown). To verify that the increased level of Bcl-xL in response to DAMGO in acute colitis reflected a decrease in apoptosis, we examined the levels of caspase 3 and 7 as marker of apoptosis [40]. This study showed that both caspase enzymes were significantly increased in DSS-induced colitis, the increased levels were reversed to controls by DAMGO and CTAP antagonized DAMGO-induced suppression of both caspase 3 and 7 (Fig. 7).

In our study, mice treated with 3% DSS showed a significant increase of NF-kB ($p<0.01$) compared to control mice. NF-kB upregulation was prevented by DAMGO treatment ($p<0.001$), effect that was reversed by CTAP ($p<0.001$) (Fig. 6). CTAP alone did not affect the expression of NF-kB in DSS acute colitis (256.46±23.76% in DSS+CTSAP vs. 237.60±23.25% in DSS alone). By contrast, NF-kB was not upregulated in mice 4 weeks following DSS treatment and remained comparable to control levels with or without DAMGO (Fig. 6).

Effect of Bcl-family inhibitor in acute DSS-induced colitis

This set of experiments investigated the effect of an inhibitor of the Bcl family of enzymes on MPO and NF-kB as markers of inflammation in acute colitis. In mice with DSS-induced acute colitis treated with DAMGO and Bcl inhibitor, the levels of MPO were similar to the levels observed in mice that received DSS without DAMGO treatment, whereas Bcl alone did not affect MPO levels in acute colitis (Fig, 7). Similarly, the levels of NBF-kB in mice with DSS-induced acute colitis treated with DAMGO and Bcl inhibitor were comparable to the levels in DSS alone. Bcl inhibitor alone did not affect the expression of NF-kB in DSS mice (Fig. 7).

DISCUSSION

This study shows that DSS-induced acute colitis, characterized by high DAI, pronounced neutrophil infiltration, tissue damage and increased expression of cytokines and NF-kB, was markedly improved by μOR activation, which significantly reduced inflammatory indexes and decreased the levels of inflammatory cytokines and NF-kB. DAMGO's beneficial effects were associated with upregulation of the antiapoptotic factor, Bcl-xL, were mediated by peripheral μORs, since DAMGO does not readily pass the blood brain barrier [32], and were prevented by the selective and potent μOR antagonist, CTAP [31], which is likely to act directly on DAMGO-activated peripheral μORs, since CTAP administered i.c.v. does not block the effect of systemic DAMGO [32]. Acute colitis also triggered the induction of μOR mRNA. By contrast, 4 weeks following DSS administration, symptoms subsided as indicated by the weight gain and normalization of DAI suggesting remission, independently of the presence or absence of DAMGO treatment. However, inflammatory indexes were still significantly elevated compared to controls, though they were much less pronounced than in the acute phase of colitis, indicating persistence of inflammation. NF-kB was not activated and μOR mRNA was back to normal levels. DAMGO-induced activation of μORs did not affect any of the inflammatory indexes nor did alter Bcl-xL expression. These findings suggest that activation of μOR ameliorates acute colitis by reducing the inflammatory response and cytokine expression through a mechanism involving the upregulation of the antiapoptotic regulator factor, Bcl-xL, and the suppression of the NF-kB inflammatory pathway. By contrast, DAMGO does not maintain its beneficial effect during the delayed phase of colitis, which coincided with the absence of DAMGO-induced Bcl-xL upregulation, and was characterized by normal levels of NF-kB and μOR mRNA. The normalization of NF-kB expression and μOR mRNA levels and the reduction of inflammatory indexes and cytokines following DAMGO treatment during the acute phase of colitis might play a role in the reduced level of inflammation observed in delayed colitis. Whether the reduced acute inflammation resulting from μOR activation influences the inability of DAMGO to further affect the inflammatory responses in the delayed phase of experimental colitis remains to be elucidated.

The reduction of pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 in response to μOR activation observed in our model of acute colitis is consistent with the reported inhibitory effect of μOR agonists on cytokines in a different experimental model of colitis, and with the cytokine increased expression in animals with genetic deletion of μOR gene [20]. DAMGO also reversed the slight, not significant increase in IL-10 expression induced by DSS. IL-10 is a cytokine produced by T-helper2 cells, which has been regarded as a downregulator of inflammatory response and an inhibitor of pro-inflammatory cytokine production [41]. However, other studies do not confirm the protective effect of IL-10 in inflammation [42]. The role of this cytokine in this model of acute colitis highly dependent on the innate immune response remains to be established. Interestingly, in addition to regulate immune response, μORs are also modulated by cytokines, supporting the concept of a bidirectional interaction and regulation between the μORs and the immune system [43, 44].

The upregulation of μOR mRNA in our model of acute colitis extend previous findings in other models of intestinal inflammation [20, 21] and is consistent with studies in humans showing increased expression of μOR mRNA in colonic specimens of patients with active IBD compared to inactive disease [22]. The effect of DAMGO on the inflammatory response in colitis correlated with the level of expression of μOR mRNA. Indeed, DAMGO had a beneficial effect in the presence of μOR mRNA upregulation in acute colitis, whereas it did not have any effect when μOR mRNA levels returned to normal in the delayed phase of colitis. μORs are abundant in the gastrointestinal tract, where they are localized to enteric neurons and inflammatory cells [7–9, 45]. Since inflammatory cells are substantially increased in acute inflammation, it is reasonable to suggest that they account for most of the upregulation of μOR mRNA and that μORs on these cells mediate DAMGO beneficial effects on inflammation, though an involvement of μORs expressed by enteric neurons cannot be ruled out on the basis of our data. Our hypothesis that the increased expression of μOR mRNA is primarily due to the increased number of inflammatory cells is supported by data showing increased number of lamina propria mononuclear cells immunoreactive for μOR in specimens of patients with active IBD compared to inactive disease and controls, whereas the density of μOR immunoreactive neurons did not change [22]. Upregulation of endogenous opioids such as β-endorphin and enkephalin, which have high affinities for μOR [46] and are also expressed in immune cells of the gut mucosa [18, 47], has been reported in experimental colitis and IBD. Indeed, β-endorphin expression is increased during the DSS-induced colitis, but in the delayed and not acute phase of colitis [48] and enkephalin has been reported to be increased in colonic specimens of patients with active Crohn's and ulcerative colitis, where it correlated with the disease activity index [49]. Though these data cannot be easily compared because of differences in species and methodology, changes in the expression of the opioid system represent a common factor in intestinal inflammation. Finally, it is tempting to speculate that the lack of effect of increasing doses of DAMGO on the inflammatory response in acute colitis is the result of μOR desensitization, a mechanism that reduces cellular response, which is induced by continuous receptor activation [50–52]. It should also be pointed out that we could not exclude that DSS affected the efficiency of DAMGO to activate μOR or μOR signaling, though this seems unlikely.

The increased expression of NF-kB, an important regulator of immune response, in the acute phase of colitis is consistent with its role in the onset of experimental colitis [53]. Since NF-kB potentiates inflammatory response by inducing cytokine production [54] and DAMGO prevented NF-kB upregulation, we can propose that the amelioration of DSS colitis and reduction of cytokine expression induced by μOR activation are mediated by DAMGO-induced suppression of NF-kB. NF-kB has also been reported to modulate μOR expression via production and release of the pro-inflammatory cytokine TNFα [55], further supporting the concept of bidirectional interactions between the μOR and the immune system. The Bcl-xL upregulation induced by DAMGO in acute colitis supports an antiapoptotic effect of μOR activation, which is reinforced by the concomitant reduction of caspase 3 and 7, indexes of apoptosis, induced by DAMGO. This concept is further supported by the suppression of the inhibitory effect of DAMGO on MPO and NF-kB in acute colitis with a Bcl family inhibitor. These findings are in line with the reported antiapoptotic properties of μORs in hepatocytes as a mechanism underlying the protective effect of μOR agonists in

acute hepatitis in mouse [23]. Bcl-xL is a zinc finger protein of the *bcl* family involved in cell death regulation, which binds with and sequesters proapoptotic effectors, such as Bax, Raf-1 kinase or other factors, forming heterodimers that promote cell survival and protect tissue from injury-induced cell death [27, 28]. DAMGO-induced upregulation of Bcl-xL paralleled the suppression of NF-kB expression suggesting that the reduction of the acute inflammatory response is triggered by an interplay between Bcl-xL and NF-kB modulated by μOR activation.

The absence of clear symptoms of inflammation 4 weeks following DSS cycle suggests remission, but the presence of significant mucosal damage, cellular infiltration and cytokine expression is indicative of persistent inflammation. A previous study using the same protocol of one cycle of DSS followed by observation up to 4 weeks, showed increasing levels of immune cell infiltration and levels of cytokines that remained elevated throughout the 4 weeks following the end of DSS reaching higher peaks compared to the acute phase, whereas the histological damage and symptoms decreased, a condition that has been regarded as chronic colitis [35]. We have not analyzed periods in between the end of DSS treatment and the 4 weeks following the induction of colitis, thus we cannot establish whether the lower, but significant delayed inflammation detected at 4 weeks represents a transition from acute colitis to remission or a progression toward a chronic inflammation following transient remission.

Interestingly, in a previous study using DSS as a model of colitis, the administration of the opioid receptor antagonist, naltrexone reduced the inflammatory response [56]. Naltrexone is a universal opioid antagonist that acts on all three opioid receptors, ∂OR and kOR in addition to the μORs, which are all expressed by enteric neurons and immune cells, and play a role in modulating the inflammatory responses [9, 17, 57]. However, unlike μORs that are immunosuppressive, ∂ORs are likely to be pro-inflammatory [17]. Furthermore, naltrexone passes the blood-brain barrier thus acting both peripherally and centrally [58]. By contrast, DAMGO, which was used in our and previous studies [20, 24], is a selective μOR agonist that predominantly acts on peripheral μORs because it does not pass the brain-blood barrier. These observations reconcile the apparent discrepancies reported by these studies and raise the possibility that different components of the opioid system interact in modulating inflammatory processes, which warrants further investigation.

In conclusion, our study shows that peripheral μOR activation ameliorates the acute, but not the delayed phase, of DSS-induced colitis. This effect is likely to be dependent on Bcl-xL activation and NF-kB suppression thus resulting in reduction of pro-inflammatory cytokines and inflammatory response. Our findings support previous observations suggesting that activation of μOR could be explored as a therapeutic target for acute inflammation [20, 22], but the lack of effect during the delayed phase of colitis does not support a role of μOR agonists in maintaining remission.

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REFERENCES

1. Pasternak GW. Pharmacological mechanisms of opioid analgesics. Clin. Neuropharmacol. 1993; 16:1–18. [PubMed: 8093680]
2. Simon EJ. Opioid receptors and endogenous opioid peptides. Med. Res. Review. 1991; 11:357–374.
3. Mansour A, Fox CA, Akil H, Watson SJ. Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci. 1995; 18:22–29. [PubMed: 7535487]
4. Mansour A, Khachaturian H, Lewis ME, et al. Anatomy of CNS opioid receptors. Trends in neurosciences. 1988; 11:308–314. [PubMed: 2465635]
5. Contet C, Kieffer BL, Befort K. Mu opioid receptor: a gateway to drug addiction. Current opinion in neurobiology. 2004; 14:370–378. [PubMed: 15194118]
6. Matthes HW, Maldonado R, Simonin F, et al. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ-opioid receptor gene. Nature. 1996; 383:819–823. [PubMed: 8893006]
7. Bagnol D, Mansour A, Akil H, Watson SJ. Cellular localization and distribution of the cloned mu and kappa opioid receptors in rat gastrointestinal tract. Neuroscience. 1997; 81:579–591. [PubMed: 9300443]
8. Ho A, Lievore A, Patierno S, et al. Neurochemically distinct classes of myenteric neurons express the μ-opioid receptor in the guinea pig ileum. J Comp Neurol. 2003; 458:404–411. [PubMed: 12619074]
9. Sternini C, Patierno S, Selmer IS, Kirchgessner A. The opioid system in the gastrointestinal tract. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2004; 16(Suppl 2):3–16. [PubMed: 15357847]
10. Holzer P. Opioid receptors in the gastrointestinal tract. Regul Pept. 2009; 155:11–17. [PubMed: 19345246]
11. Kromer W. Endogenous and exogenous opioids in the control of gastrointestinal motility and secretion. Pharmacological reviews. 1988; 40:121–162. [PubMed: 3070578]
12. Wood JD, Galligan JJ. Function of opioids in the enteric nervous system. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2004; 16(Suppl 2):17–28. [PubMed: 15357848]
13. Chuang TK, Killam KF Jr, Chuang LF, et al. Mu opioid receptor gene expression in immune cells. Biochem Biophys Res Commun. 1995; 216:922–930. [PubMed: 7488213]
14. Madden JJ, Whaley WL, Ketelsen D. Opiate binding sites in the cellular immune system: expression and regulation. Journal of neuroimmunology. 1998; 83:57–62. [PubMed: 9610673]
15. Stefano GB, Scharrer B, Smith EM, et al. Opioid and opiate immunoregulatory processes. Crit Rev Immunol. 1996; 16:109–144. [PubMed: 8879941]
16. Eisenstein TK, Hilburger ME. Opioid modulation of immune responses: effects on phagocyte and lymphoid cell populations. Journal of neuroimmunology. 1998; 83:36–44. [PubMed: 9610671]
17. McCarthy L, Wetzel M, Sliker JK, et al. Opioids, opioid receptors, and the immune response. Drug Alcohol Depend. 2001; 62:111–123. [PubMed: 11245967]
18. Sharp BM, Roy S, Bidlack JM. Evidence for opioid receptors on cells involved in host defense and the immune system. Journal of neuroimmunology. 1998; 83:45–56. [PubMed: 9610672]
19. Holzer P, Ahmedzai SH, Niederle N, et al. Opioid-induced bowel dysfunction in cancer-related pain: causes, consequences, and a novel approach for its management. J Opioid Manag. 2009; 5:145–151. [PubMed: 19662924]
20. Philippe D, Dubuquoy L, Groux H, et al. Anti-inflammatory properties of the mu opioid receptor support its use in the treatment of colon inflammation. The Journal of clinical investigation. 2003; 111:1329–1338. [PubMed: 12727924]

21. Pol O, Alameda F, Puig MM. Inflammation enhances mu-opioid receptor transcription and expression in mice intestine. Molecular pharmacology. 2001; 60:894–899. [PubMed: 11641416]
22. Philippe D, Chakass D, Thuru X, et al. Mu opioid receptor expression is increased in inflammatory bowel diseases: implications for homeostatic intestinal inflammation. Gut. 2006; 55:815–823. [PubMed: 16299031]
23. Chakass D, Philippe D, Erdual E, et al. μ Opioid receptor activation prevents acute hepatic inflammation and cell death. Gut. 2007; 56:974–981. [PubMed: 17299060]
24. Saccani F, Anselmi L, Jaramillo I, et al. Protective role of mu opioid receptor activation in intestinal inflammation induced by mesenteric ischemia/reperfusion in mice. J Neurosci Res. 2012; 90:2146–2153. [PubMed: 22806643]
25. Kim TW, Seo JN, Suh YH, et al. Involvement of lymphocytes in dextran sulfate sodium-induced experimental colitis. World journal of gastroenterology : WJG. 2006; 12:302–305. [PubMed: 16482634]
26. Boise LH, Gonzalez-Garcia M, Postema CE, et al. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell. 1993; 74:597–608. [PubMed: 8358789]
27. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nature reviews. Cancer. 2002; 2:647–656.
28. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nature reviews. Molecular cell biology. 2014; 15:49–63.
29. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annual review of immunology. 1994; 12:141–179.
30. Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. Oncogene. 2006; 25:6680–6684. [PubMed: 17072321]
31. Kramer TH, Shook JE, Kazmierski W, et al. Novel peptidic mu opioid antagonists: pharmacologic characterization in vitro and in vivo. The Journal of pharmacology and experimental therapeutics. 1989; 249:544–551. [PubMed: 2566679]
32. Al-Khrasani M, Spetea M, Friedmann T, et al. DAMGO and 6beta-glycine substituted 14-O-methyloxymorphone but not morphine show peripheral, preemptive antinociception after systemic administration in a mouse visceral pain model and high intrinsic efficacy in the isolated rat vas deferens. Brain Res Bull. 2007; 74:369–375. [PubMed: 17845912]
33. Abbruscato TJ, Thomas SA, Hruby VJ, Davis TP. Blood-brain barrier permeability and bioavailability of a highly potent and mu-selective opioid receptor antagonist, CTAP: comparison with morphine. The Journal of pharmacology and experimental therapeutics. 1997; 280:402–409. [PubMed: 8996221]
34. Vaillant F, Merino D, Lee L, et al. Targeting BCL-2 with the BH3 mimetic ABT-199 in estrogen receptor-positive breast cancer. Cancer cell. 2013; 24:120–129. [PubMed: 23845444]
35. Melgar S, Karlsson A, Michaelsson E. Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. Am J Physiol Gastrointest Liver Physiol. 2005; 288:G1328–G1338. [PubMed: 15637179]
36. Murthy SN, Cooper HS, Shim H, et al. Treatment of dextran sulfate sodium-induced murine colitis by intracolonic cyclosporin. Digestive diseases and sciences. 1993; 38:1722–1734. [PubMed: 8359087]
37. Cattaruzza F, Cenac N, Barocelli E, et al. Protective effect of proteinase-activated receptor 2 activation on motility impairment and tissue damage induced by intestinal ischemia/reperfusion in rodents. Am J Pathol. 2006; 169:177–188. [PubMed: 16816371]
38. Anselmi L, Lakhter A, Hirano AA, et al. Expression of galanin receptor messenger RNAs in different regions of the rat gastrointestinal tract. Peptides. 2005; 26:815–819. [PubMed: 15808911]
39. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods. 2001; 25:402–408. [PubMed: 11846609]
40. Lakhani SA, Masud A, Kuida K, et al. Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science. 2006; 311:847–851. [PubMed: 16469926]

41. Markel TA, Crisostomo PR, Wairiuko GM, et al. Cytokines in necrotizing enterocolitis. Shock. 2006; 25:329–337. [PubMed: 16670633]
42. Nussler NC, Muller AR, Weidenbach H, et al. IL-10 increases tissue injury after selective intestinal ischemia/reperfusion. Annals of surgery. 2003; 238:49–58. [PubMed: 12832965]
43. Gaveriaux-Ruff C, Matthes HW, Peluso J, Kieffer BL. Abolition of morphine-immunosuppression in mice lacking the mu-opioid receptor gene. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95:6326–6330. [PubMed: 9600964]
44. Kraus J, Borner C, Giannini E, et al. Regulation of mu-opioid receptor gene transcription by interleukin-4 and influence of an allelic variation within a STAT6 transcription factor binding site. The Journal of biological chemistry. 2001; 276:43901–43908. [PubMed: 11572871]
45. Patierno S, Raybould HE, Sternini C. Abdominal surgery induces μ opioid receptor endocytosis in enteric neurons of the guinea pig ileum. Neuroscience. 2004; 123:101–109. [PubMed: 14667445]
46. Sternini, C.; Duraffourd, C.; Anselmi, L. Opioids and opioid receptors. In: Kastin, A., editor. Handbook of Biologically Active Peptides. 2013. p. 183-288.
47. Machelska H, Stein C. Immune mechanisms in pain control. Anesthesia and analgesia. 2002; 95:1002–1008. table of contents. [PubMed: 12351284]
48. Valdez-Morales E, Guerrero-Alba R, Ochoa-Cortes F, et al. Release of endogenous opioids during a chronic IBD model suppresses the excitability of colonic DRG neurons. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2013; 25:39–46. e34. [PubMed: 22963585]
49. Owczarek D, Cibor D, Mach T, et al. Met-enkephalins in patients with inflammatory bowel diseases. Advances in medical sciences. 2011; 56:158–164. [PubMed: 22112433]
50. Bohm S, Grady EF, Bunnett NW. Mechanisms attenuating signaling by G protein-coupled receptors. Biochem J. 1997; 322:1–18. [PubMed: 9078236]
51. Duraffourd C, Kumala E, Anselmi L, et al. Opioid-Induced Mitogen-Activated Protein Kinase Signaling in Rat Enteric Neurons following Chronic Morphine Treatment. PloS one. 2014; 9:e110230. [PubMed: 25302800]
52. Sternini C. Receptors and transmission in the brain-gut axis: potential for novel therapies. III. μ opioid receptors in the enteric nervous system. Am. J. Physiol. 2001; 281:G8–G15.
53. Funakoshi T, Yamashita K, Ichikawa N, et al. A novel NF-kappaB inhibitor, dehydroxymethylepoxyquinomicin, ameliorates inflammatory colonic injury in mice. Journal of Crohn's & colitis. 2012; 6:215–225.
54. Al-Mohanna F, Saleh S, Parhar RS, Collison K. IL-12-dependent nuclear factor-kappaB activation leads to de novo synthesis and release of IL-8 and TNF-alpha in human neutrophils. Journal of leukocyte biology. 2002; 72:995–1002. [PubMed: 12429722]
55. Tegeder I, Geisslinger G. Opioids as modulators of cell death and survival--unraveling mechanisms and revealing new indications. Pharmacological reviews. 2004; 56:351–369. [PubMed: 15317908]
56. Matters GL, Harms JF, McGovern C, et al. The opioid antagonist naltrexone improves murine inflammatory bowel disease. J Immunotoxicol. 2008; 5:179–187. [PubMed: 18569389]
57. Waldhoer M, Bartlett SE, Whistler JL. Opioid receptors. Annual review of biochemistry. 2004; 73:953–990.
58. Raynor K, Kong H, Yasuda K, et al. Pharmacological characterization of the clones k, ∂ and μ opioid receptors. Molecular pharmacology. 1994; 45:330–334. [PubMed: 8114680]

Key Messages

- μ opioid receptors (μORs) are expressed by a variety of cell types, i.e. neurons and immunocytes, and are known to mediate immune response, in addition to analgesia and gastrointestinal functions, including motility, secretion, electrolyte balance and absorption.
- In this paper, we tested whether activation of peripheral μORs affects the immune activation/inflammatory response as well as disease activity index in the acute vs. the delayed phase of colitis in mouse using the dextran sodium sulfate (DSS) model of inflammation that is highly dependent on innate immunity.
- Colitis was induced in C57BL/6J adult mice using a cycle of 3% DSS in drinking water for 5 days. Controls received only drinking water. The effect of a selective, peripherally-acting, μOR agonist on disease activity index, histological damage, apoptosis, inflammatory indexes, nuclear factor kB (NF-kB), the antiapoptotic factor, Bcl-xL and μOR expression was evaluated at the end of DSS treatment and 4 weeks following DSS. Quantitative RT-PCR and Western blot were used.
- Our data showed that μOR agonist reduced the disease activity (e.g. body weight, diarrhea and fecal blood), tissue damage, apoptosis, inflammatory indexes, cytokine levels and NF-kB expression, and increased Bcl-xL levels in the acute phase of colitis but had no effect on the delayed phase, in which inflammatory indexes and cytokines are still elevated except for NF-kB. These findings suggest that activation of μORs in the gut ameliorates the acute, but not the delayed phase, of DSS-induced colitis, through Bcl-xL activation and NF-kB suppression. μOR agonists acting peripherally could be explored as a therapeutic approach for acute inflammation, but not for maintenance of remission.

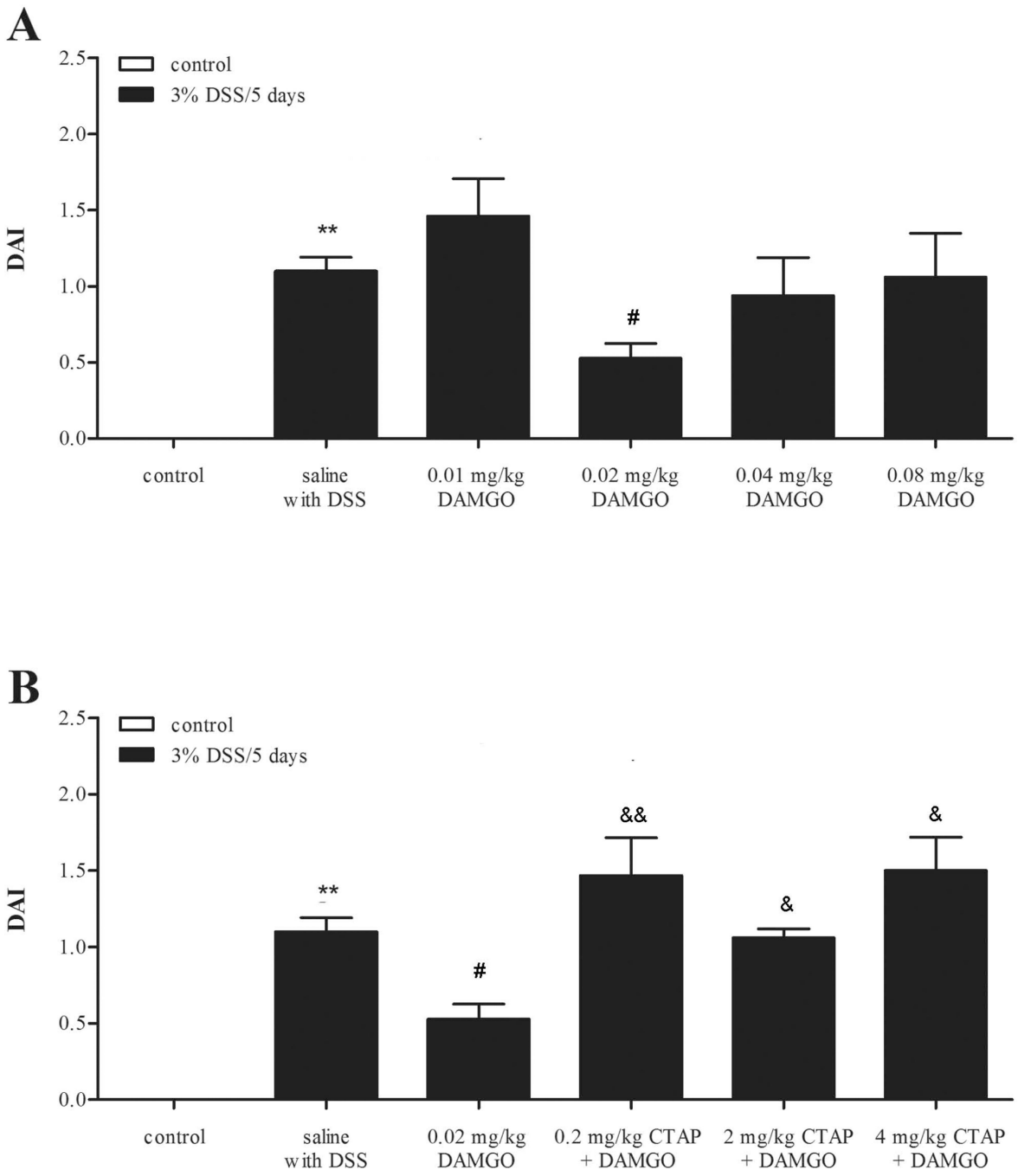

Figure 1. Effect of DAMGO on disease activity index in intestinal inflammation

Disease activity index (DAI) in control mice and mice with DSS-induced colitis untreated or treated with 0.01, 0.02, 0.04 or 0.08 mg kg^{-1} × day of DAMGO (5 mice in each group except for the group with DSS only that included 17 mice and the group treated with DAMGO 0.02 mg kg^{-1} × day that included 11 mice) (A), and colitis treated with DAMGO 0.02 mg plus 0.2, 2 or 4 mg kg^{-1} CTAP (5 mice each group) (B). DAI was significantly elevated in DSS treated mice compared to control (**p<0.01) (A and B). A: DAMGO at 0.02 mg significantly reduced DAI compared to DSS alone (#p<0.05 DAMGO vs. DSS),

whereas lower and higher doses did not have significant effects. B: The effect of DAMGO at 0.02 mg on DAI was reversed by CTAP (&p<0.05 and && p<0.01 DAMGO+CTAP vs. DAMGO). Columns represent mean±SEM of DAI values obtained by averaging weight loss, stool consistency and bleeding scores.

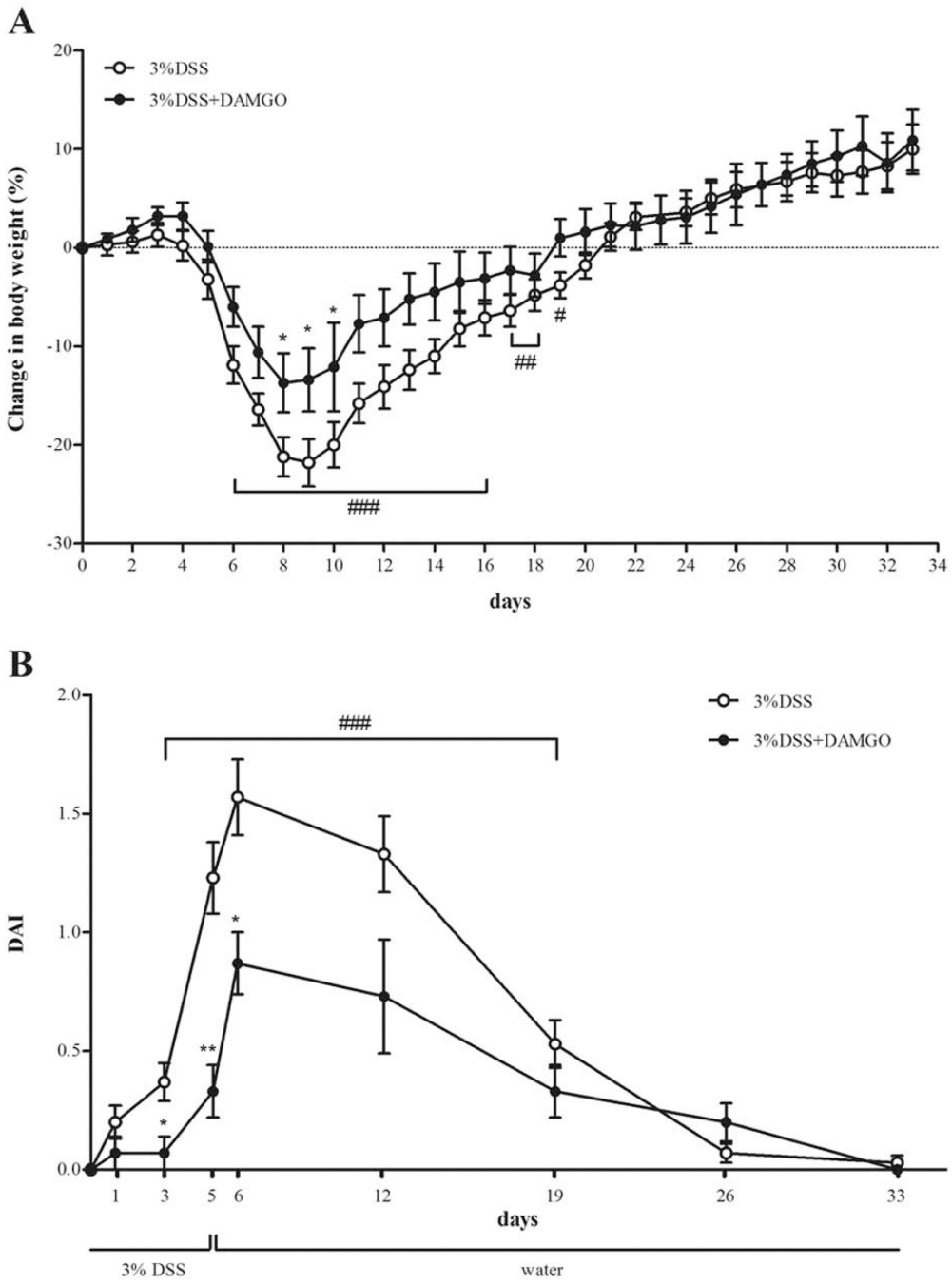

Figure 2. Time course changes of body weight and DAI in DSS-induced colitis

Changes in body weight (A) and DAI (B) in mice during and following the induction of colitis. Open circles represent results in mice exposed to 3% DSS for 5 days followed by drinking water alone for four weeks (n=10). Closed circles represent changes in mice treated with 0.02 mg kg^{-1} × day DAMGO during DSS administration and for two weeks following DSS treatment followed by two weeks of water (n=5). A: There was a significant reduction in body weight in DSS treated animals from day 6 to day 19 compared to time 0 (#p<0.05, ##p<0.01, ###p<0.001); body weight progressively returned to the value at time 0 and then

increased. Body weight loss was significantly reduced by DAMGO treatment between day 8 and 10 (*p< 0.05). B: Similarly, DAI was significantly increased between days 3–19 compared to time 0 (###p<0.001) and was comparable to normal starting at day 26. DAMGO treatment significantly reduced DAI during the acute phase (day 3–6) (*p<0.05; **p<0.01) in DSS treated mice and gradually returned to normal as in animals without DAMGO treatment. Data are expressed as means ± SEM. Animals treated with DAMGO for 3 weeks following DSS and then one week of water showed the same results (not shown).

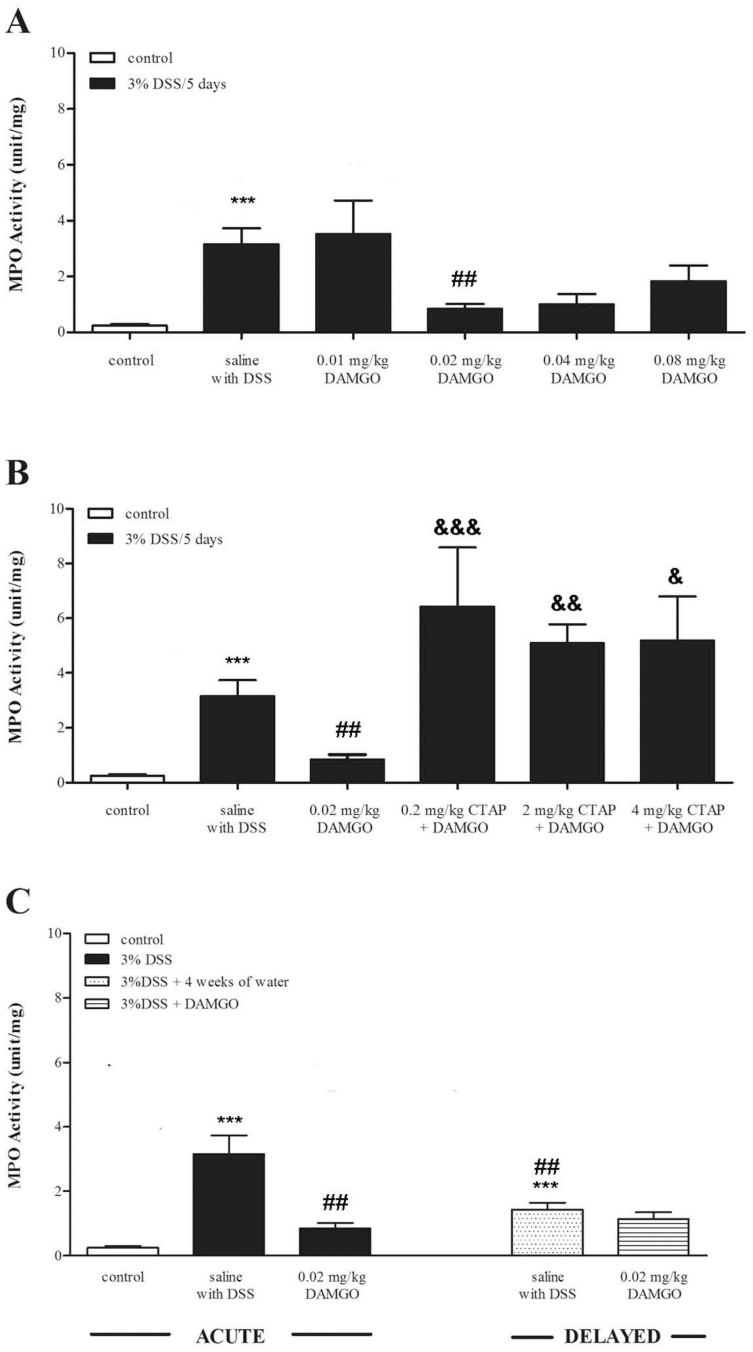

Figure 3. Myeloperoxidase (MPO) activity in DSS-induced colitis

MPO is significantly increased in DSS-colitis (***p<0.001, A,B,C) (n=10) compared to controls (n=8). A: Effect of different doses of DAMGO on MPO in DSS-induced colitis. DAMGO at 0.02 mg kg^{-1} induced the strongest and most significant decrease in MPO (##p<0.01 vs. DSS, A,B,C) (n=11); whereas the other doses of DAMGO did not affect MPO activity in DSS (DAMGO 0.01 mg/Kg, n=4; DAMGO 0.04 mg/kg, n=4; DAMGO 0.08 mg/kg, n=5). B: Effect of different doses of CTAP on 0.02 mg DAMGO on MPO in DSS-induced colitis. All concentrations of CTAP significantly reduced the inhibitory effect of

DAMGO on MPO, but the 2 mg dose had less variability compared to the other doses of CTAP (& $p<0.05$, && $p<0.01$, &&& $p<0.001$ compared to DAMGO). (CTAP 0.2 mg/Kg, n=6; CTAP 2 mg/Kg, n=10; CTAP 4 mg/Kg, n=4). Columns represent mean±SEM of MPO values. C: Comparison of the effect of DAMGO at 0.02 mg kg^{-1} on MPO in acute (n=11) and delayed phase (4 weeks following the end of DSS treatment) (n=9) of colitis. DAMGO administered daily from day 2 to 5 of DSS treatment and for 2 weeks after the end of DSS followed by 2 more weeks of drinking water (n=9) did not have any effect on MPO compared to mice that only received drinking water following DSS. MPO activity 4 weeks after the end of DSS treatment was significantly higher than in controls (***$p<0.001$), but was significantly less compared to acute DSS (##$p<0.01$). Similarly, DAMGO administered for 3 weeks followed by one more week of water (not shown) did not have any effect on MPO.

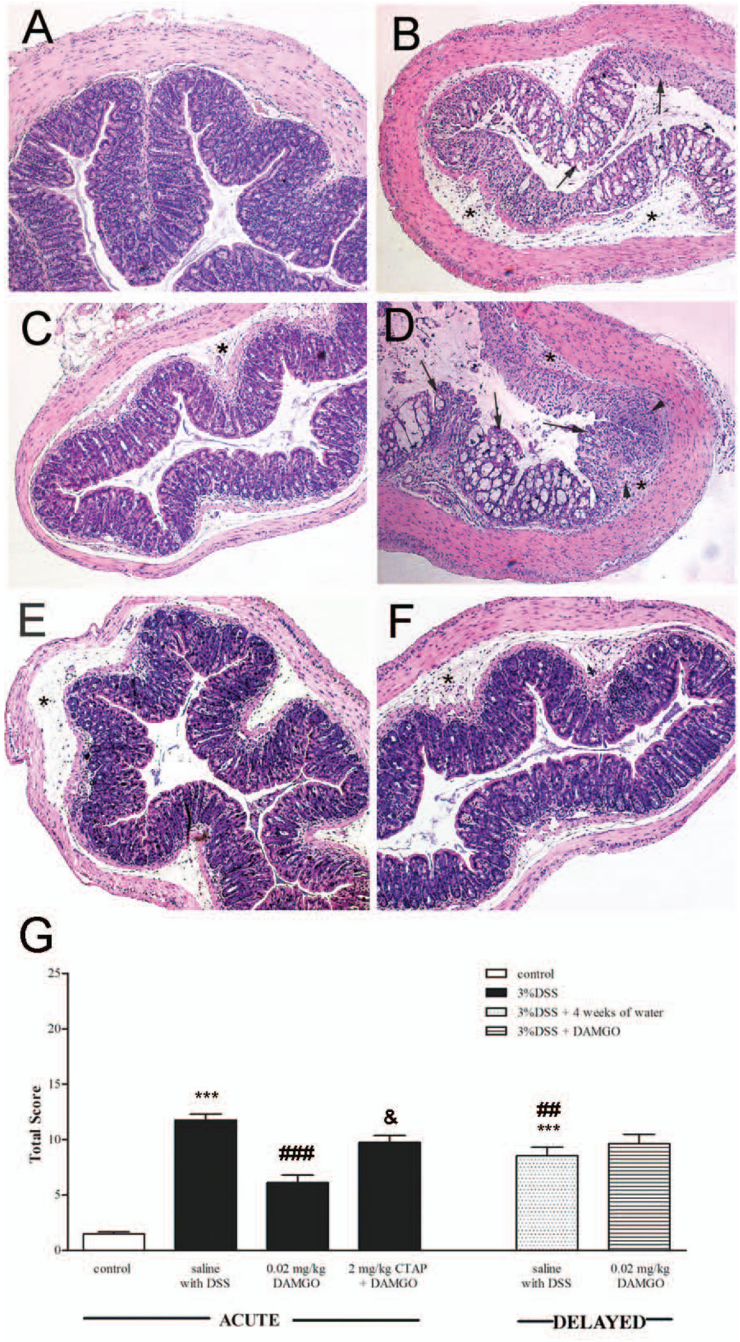

Figure 4. Histopathology of DSS colitis with or without DAMGO and CTAP vs. controls

Representative histological images from sections of the colon of normal mice (A, n=10), mice with DSS-induced colitis (B, n=13) and mice with DSS acute colitis treated with DAMGO (C, n=10) or with DAMGO plus CTAP (D, n=5). E and F show the tissue damage at 4 weeks following DSS without DAMGO treatment (E, n=10) or with DAMGO treatment (2 weeks) (F, n=5). There was prominent histological damage of the epithelium in DSS colitis (B) and DSS colitis treated with DAMGO plus CTAP (D) (arrows in B and D point to lifting of the epithelium, epithelial erosion and loss of normal crypt architecture and goblet

cell) with cellular infiltration (arrowheads in B and D) and edema in the submucosa (* in B and D) in acute colitis. The histological damage in the delayed phase of colitis was the same with or without DAMGO treatment (E, F) and predominantly included cellular infiltrates and submucosa edema (* points to submucosa edema in E and F), whereas the epithelial layer was mostly normal, but much less prominent than in acute inflammation (B). Tissue damage in acute colitis is dramatically reduced by DAMGO pretreatment (histology in C is comparable to control shown in A), except with moderate edema of the mucosa (* in C) and cellular infiltrates (not sown). CTAP reverses the effect of DAMGO and histological damage (D) is comparable to DSS alone (B). G: Quantification of histological scores shows a significant increase of total score in mice with acute DSS compared to control (***p<0.001), a significant reduction by DAMGO (###p<0.001 vs. DSS), which was reversed by CTAP (&p<0.05 vs. DAMGO). Total score was still significantly higher 4 weeks following DSS treatment compared to control (***p<0.001), but significantly lower compared to acute DSS (*##p<0.01) and was not significantly affected by DAMGO treatment. Columns represent mean±SEM.

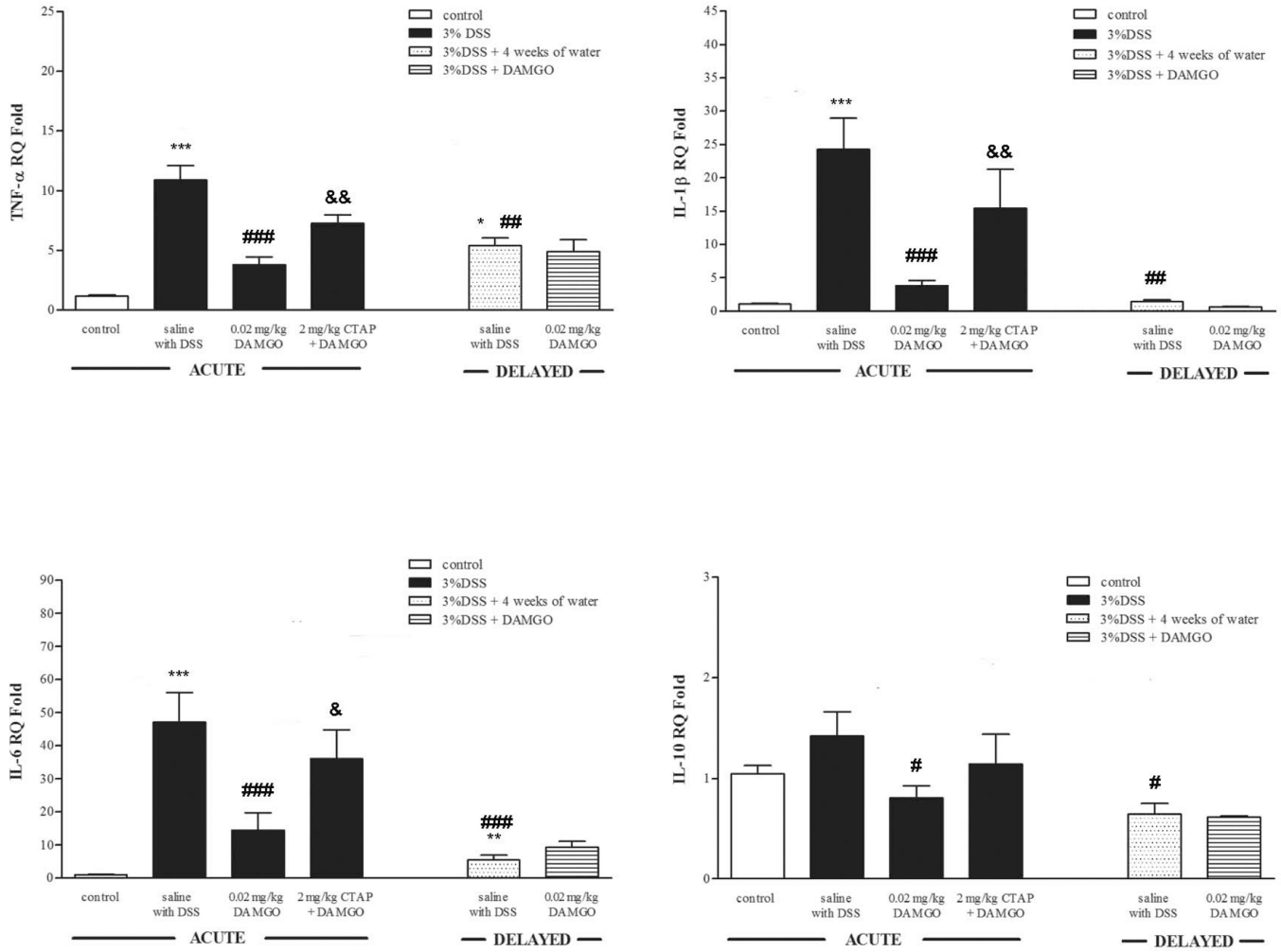

Figure 5.

Effect of DAMGO on cytokine levels in DSS colitis. In mice with acute colitis (n=17), mRNA levels of pro-inflammatory cytokine TNF-α, IL-1β, and IL-6 were significantly higher compared to control (***p<0.001) (n=9), whereas IL-10 was only slightly, but not significantly, elevated. DAMGO (0.02 mg kg^{-1} s.c.) treatment (n=10) significantly reduced TNF-α, IL-1β, IL-6 (###p<0.001) and IL-10 (#p<0.05) mRNA expression compared to DSS only. DAMGO inhibitory effect on TNF-α, IL-1β, IL-6 mRNA levels was reversed by CTAP (n=6). Mice at 4 weeks after DSS treatment (n=5) showed a significant increase of TNF-α and IL-6 expression compared to control (*p<0.05, **p<0.01); however all cytokines were significantly lower at 4 weeks compared to acute colitis (#p<0.05, ##p<0.01, ###p<0.001). DAMGO (n=5) did not affect cytokine mRNA levels at 4 weeks following DSS. All values are expressed as mean±SEM.

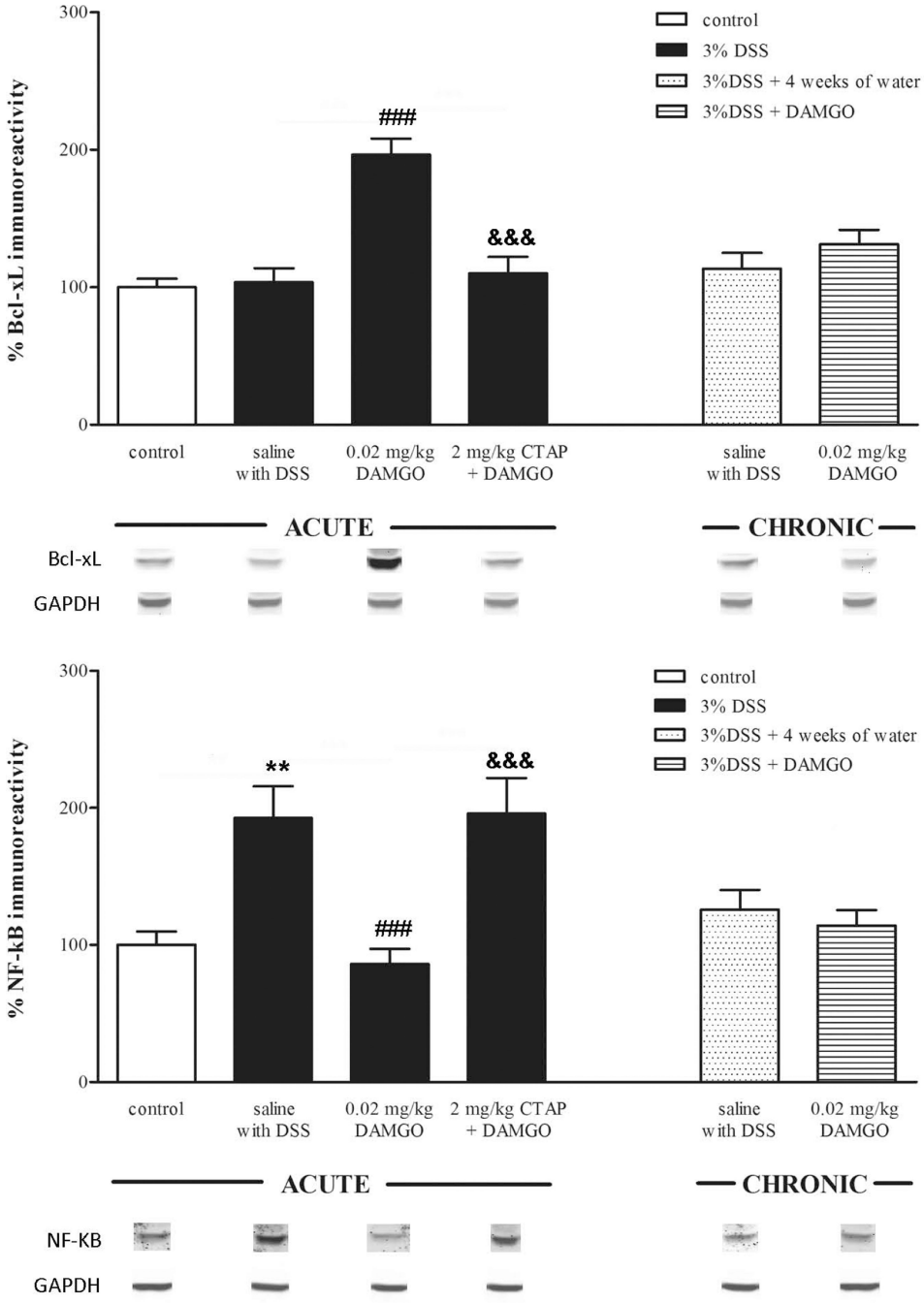

Figure 6. Bcl-xL and NF-kB protein expression in DSS colitis

Western blot analysis of Bcl-xL and NF-kB immunoreactivity in colon from control mice and mice with acute colitis or at 4 weeks following DSS. Bcl-xL and NF-kB immunoreactivity levels were normalized to the housekeeping protein GADPH. Results represent the percentage variation of Bcl-xL and NF-kB compared to control. DAMGO induced a significant increase in the levels of Bcl-xL immunoreactivity in acute colitis (###$p<0.001$ vs. control and DSS alone), but not at 4 weeks. The effect of DAMGO was significantly reversed by CTAP (&&&$p<0.001$). NF-kB immunoreactivity levels were

significantly increased in acute colitis (**p<0.01), but not at 4 weeks. In acute colitis, DAMGO significantly reduced NF-kB (###p<0.001 vs. DSS alone), effect that was reversed by CTAP (&&&p<0.001). DAMGO did not affect NF-kB at 4 weeks. All values are expressed as mean±SEM. N=5 per group. Representative immunoblots of Bcl-xL and NF-kB immunoreactivities are shown at the bottom of each histogram. GAPDH served as housekeeping protein to verify that the same amount of proteins was loaded on each gel.

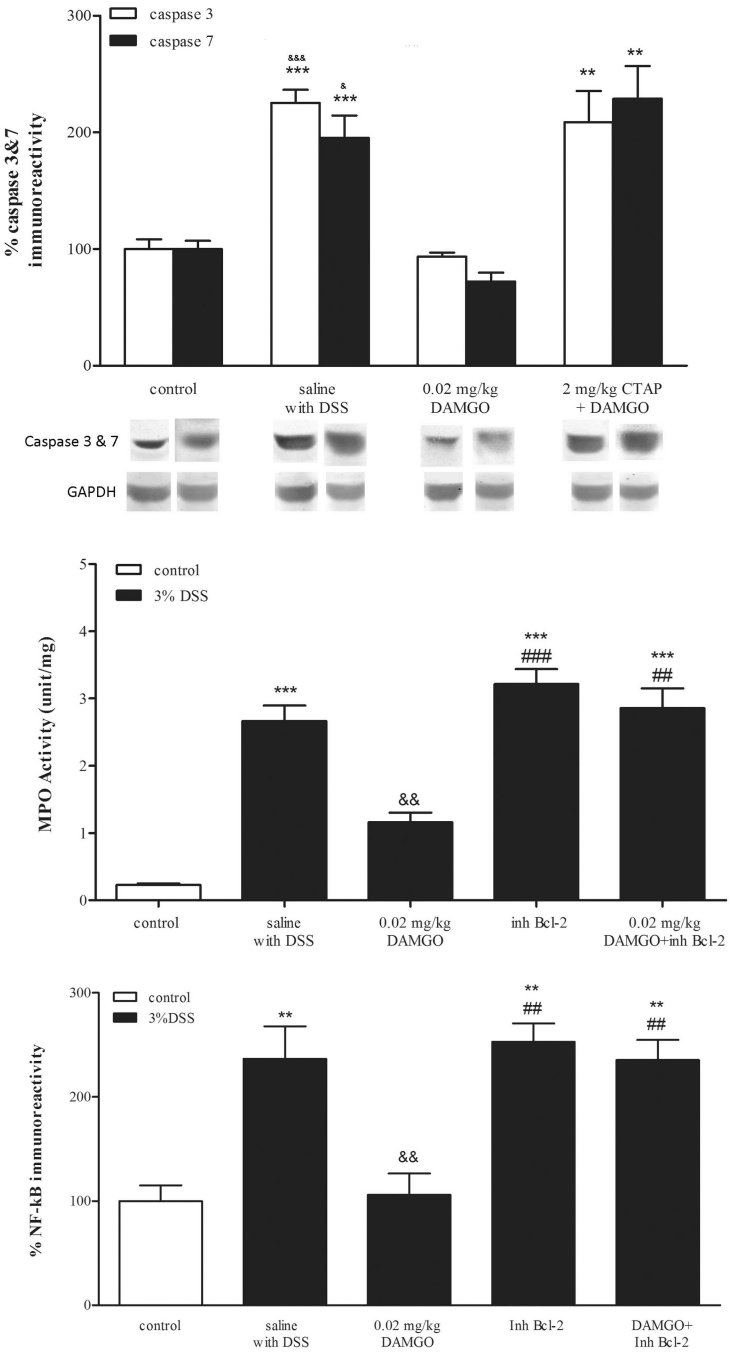

Figure 7.

Caspase 3 and 7 in acute DSS colitis with and without DAMGO (A) and the effect of Bcl family inhibitor on MPO (B) and NF-kB (C) with and without DAMGO. A: DSS induces a significant increase in the levels of caspase 3 and 7 (&p<0.05, &&p<0.001 vs. control), which is significantly reduced by DAMGO (***p<0.001). CTAP reverses the effect of DAMGO (**p<0.01 vs. DAMGO). N=4 per group. Representative immunoblots of caspase 3 and 7 immunoreactivities are shown at the bottom of the histogram in A. GAPDH served as housekeeping protein to verify that the same amount of proteins was loaded on each gel.

B and C show the significant increase in MPO (B) and NF-kB (C) induced by DSS (**p<0.01, ***p<0.001), which is reversed by DAMGO (&&p<0.01 vs. DSS). Injection of an inhibitor of Bcl-2 family suppresses the effect of DAMGO on MPO (B) and NF-kB (C) (##p<0.01 vs. DAMGO; **p<0.01, ***p<0.001 vs. controls). Bcl-2 inhibitor alone does not affect the increase in MPO and NF-kB induced by DSS (**p<0.01, ***p<0.001 vs. control; ##p<0.01, ###p<0.001 vs. DAMGO treatment). Controls, n=8; DSS, n=10; DSS+DAMGO, n=11; DSS+Bcl-2 inhibitor, n=4; DSS+DAMGO+ Bcl-2 inhibitor, n=4.