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Protective role of μ opioid receptor activation in intestinal inflammation induced by mesenteric ischemia/reperfusion in mice

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Abstract

Intestinal ischemia is a clinical emergency with high morbidity and mortality. We investigated whether activation of μ opioid receptor (μ OR) protects from the inflammation induced by intestinal ischemia and reperfusion (I/R) in mice. Ischemia was induced by occlusion of the superior mesenteric artery (45 min) and followed by reperfusion (5 hours). Sham Operated (SO) and normal (N) mice served as controls. Each group received subcutaneously: (1) saline solution; (2) the μ OR selective agonist, [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO) (0.01 mg.kg⁻¹); (3) DAMGO and the selective μ OR antagonist [H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂] (CTAP) (0.1 mg.kg⁻¹) or (4) CTAP alone. I/R induced intestinal inflammation as indicated by histological damage and the significant increase in myeloperoxidase (MPO) activity, index of tissue neutrophil accumulation. TNF- α and IL-10 mRNA levels were also increased in I/R mice compared to SO. DAMGO significantly reduced tissue damage, MPO activity and TNF- α mRNA levels in I/R and these effects were reversed by CTAP. By contrast, DAMGO did not modify IL-10 mRNA levels and gastrointestinal transit. DAMGO effects are receptor-mediated and are likely due to activation of peripheral μ ORs since it does not readily cross the blood brain barrier. These findings suggest that activation of peripheral μ OR protects from the inflammatory response induced by I/R through a pathway involving the pro-inflammatory cytokine, TNF- α . Reduction of acute inflammation might prevent I/R complications, including motility impairment, which develop at a later stage of reperfusion and are likely due to inflammatory cell infiltrates.

Keywords

intestinal injury; myeloperoxidase activity; cytokines; DAMGO

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INTRODUCTION

Intestinal ischemia is a life-threatening emergency with high mortality rate that ranges from 30% to 90% depending on the etiology (Herbert and Steele 2007; Martinez and Hogan 2004; Paterno and Longo 2008). It can occur in several clinical-surgical conditions, including abdominal surgery for aortic aneurysm, small bowel transplantation, cardiopulmonary bypass, strangulated hernias and neonatal necrotizing enterocolitis (Mallick et al. 2004). Intestinal ischemia, a consequence of reduction of tissue oxygenation, induces progressive tissue damage and necrosis, which is paradoxically aggravated by restoration of the blood flow or reperfusion (Cerqueira et al. 2005).

Intestinal ischemia and reperfusion (I/R) trigger a cascade of events, including increase in vascular permeability, alteration in absorption, activation and adhesion of polymorphonuclear neutrophils, release of pro-inflammatory substances, and bacterial translocation. Mediators released from mast cells and macrophages, which normally reside in close proximity to post capillary venules, aggravate the inflammatory response induced by I/R (Carden and Granger 2000). Furthermore, the overproduction of inflammatory cytokines and translocation of enteric bacteria can induce remote tissue injury and systemic inflammatory response syndrome, which can progress to multiple organs failure (Berg 1999; Cerqueira et al. 2005; Cuzzocrea et al. 2002; Granger and Korthuis 1995; Mallick et al. 2004).

Several signaling molecules have been proposed as modulators of inflammatory processes, including μ opioid receptors (μ ORs), G-protein coupled receptors that mediate a variety of biological effects such as analgesia, stress response and regulation of inflammatory processes (Chuang et al. 1995; Gaveriaux-Ruff et al. 1998; Madden et al. 1998; Stefano et al. 1996). μ ORs are activated by endogenous opioids and opiate drugs, which are commonly used in humans for pain control. μ ORs are widely expressed in the central and peripheral nervous system, including the enteric nervous system (ENS), the integrative network innervating the gastrointestinal (GI) tract (Bagnol et al. 1997; Ho et al. 2003; Sternini et al. 2004). In the GI tract, μ ORs are localized to neurons of the myenteric and submucosal plexi and immune cells (Gross and Pothoulakis 2007; Sternini et al. 2004). Interestingly, exogenous activation of μ ORs has been shown to ameliorate inflammation in experimental colitis (Philippe et al. 2003), supporting the concept that μ OR agonists might act as regulatory modulators of gut inflammatory processes.

The above observations provided the background for our hypothesis that μ ORs activation might attenuate the inflammatory response induced by intestinal ischemia and reperfusion. In order to test our hypothesis, we used a model of mild mesenteric ischemia developed in mice by reversible occlusion of the superior mesenteric artery (SMA) for 45 min followed by 5 hours of reperfusion (Ballabeni et al. 2002; Barocelli et al. 2006; Bertoni et al. 2007; Calcina et al. 2005; Cattaruzza et al. 2006). Aim of the study was to investigate the effect of exogenous administration of a selective μ OR agonist in the presence or in the absence of a selective μ OR antagonist on intestinal injury induced by I/R in mice. We assessed the changes in intestinal myeloperoxidase activity, mucosal integrity, and cytokines mRNA expression, as index of local intestinal injury, and evaluated the alterations of gastrointestinal motility and μ OR mRNA expression in I/R mice in comparison with normal or sham-operated mice.

MATERIALS AND METHODS

Animal care and procedures were in accordance with the National Institute of Health recommendations for the humane use of animals. All experimental procedures were

reviewed and approved by the Animal Research Committee of the University of California, Los Angeles. Experiments were performed on female adult C57BL/6 mice (20–25g; Charles River Laboratories, Montreal, QC, Canada) that were housed under standard conditions and fasted, with free access to water, 12 hours before the experimental procedures.

Ischemia/reperfusion procedure

Three groups of 30–36 mice each were investigated. One group underwent intestinal ischemia followed by reperfusion (I/R), one group underwent sham operation (SO) and one group comprised normal animals (N) that did not undergo any type of surgery. Animals that underwent surgery were anaesthetized with Nembutal (50 mg kg⁻¹ i.p.). Following abdominal laparotomy the small bowel was retracted to the left and the SMA was identified and temporarily occluded using a micro-vascular clip for 45 min. By gently removing the clip, the reperfusion was allowed for a period of 5 hours and the abdominal cavity was closed by a two-layer suture. SO mice underwent the same surgical procedure without the occlusion of the SMA. Each group (I/R, SO and N) was subdivided in four subgroups, each receiving one of the following subcutaneous treatments: saline solution (1ml kg⁻¹, 20 min before the beginning of the ischemic period); [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO; 0.01 mg kg⁻¹, 20 min before ischemic period and 2h after the beginning of reperfusion); [H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂] (CTAP; 0.1 mg kg⁻¹, 35 min before beginning of the ischemia) or co-administration of DAMGO and CTAP. The dose of DAMGO was selected on the basis of a pilot study, in which we measured MPO activity in intestinal tissue of I/R mice in response to subcutaneous administration of increasing concentrations of DAMGO (0.001, 0.01 and 0.1 mg/Kg) vs. saline (5–6 animals per group) 20 min before ischemia and 2 hours after the beginning of reperfusion. I/R mice treated with DAMGO 0.001 mg/kgx2 had an MPO activity of 0.90±0.31 mU/mg tissue; those treated with DAMGO 0.01 mg/kgx2 had 0.43±0.13 mU MPO/mg tissue; those treated with DAMGO 0.1 mg/kgx2, had 0.92±0.13 mU MPO/mg tissue; whereas I/R mice that receive saline only without DAMGO had 1.2940 ± 0.2 mU MPO/mg tissue. This pilot study shows that even though each dose of DAMGO reduced MPO activity compared to saline, DAMGO 0.01 mg/kgx2 was the most effective dose that reduced MPO activity significantly (p<0.05) compared to saline only. This dose was then used for the entire study. Animals were euthanized at the end of the experiments by injection of overdose of Nembutal (100 mg kg⁻¹ i.p.) followed by thoracotomy.

Assessment of inflammation

Specimens of the distal ileum were collected from each group of animals at the end of the reperfusion period to determine the level of tissue damage. Following overnight fixation in 10% formalin, samples of ileum were embedded in paraffin. 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 semi-quantitative scoring system in which the following features were graded: damage of epithelium (0 morphologically normal; 1 development of subepithelial space; 2 presence of subepithelial space with moderate lifting of epithelial layer; 3 severe epithelial lifting), inflammatory cells infiltration (0 absence of infiltrate or less than five cells; 1 mild infiltration to the lamina propria; 2 moderate infiltration to the muscularis mucosae and submucosa; 3 severe transmural infiltration involving the muscle layer), extent of muscle thickening (0 normal; 1 moderate; 2 severe), edema (0 no edema; 1 zonal edema in lamina propria and submucosa; 2 diffuse edema in lamina propria and submucosa). Data were expressed as sum of scores for each feature (total score).

Gastrointestinal transit

GI transit was measured by evaluating the distribution of the non-absorbable fluorescent 70,000MW dextran ($150\mu\text{l}$, 5mg ml^{-1}) administered intragastrically by gavage after three hours and a half of reperfusion. Ninety minutes later animals were euthanized as described above. The entire GI tract, from stomach to distal colon, was excised and divided into 15 segments: stomach, small intestine (10 segments of equal length), cecum and colon (3 segments of equal length). The luminal content of each segment was collected, suspended in 1ml of distilled water, mixed vigorously and clarified by centrifugation (15min at 14,000rpm at 4°C). The supernatants of each sample were collected and the fluorescence signal was determined in triplicates by using a fluorescence plate reader (excitation wavelength 485nm and emission wavelength 528nm, FLX800 Microplate Fluorescence Reader, Bio-Tek instruments Inc., Winooski, VT, USA). GI transit was calculated as the geometric centre (GC) of the distribution of labeled dextran along the entire gastrointestinal tract (Miller et al. 1981). In some experiments, we also calculated the GC of the distribution of labeled dextran along the small intestine to test whether there were differences in transit in the segments directly affected by I/R vs. the whole GI tract.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an index of tissue neutrophil accumulation, was measured according to Krawisz's modified method (Krawisz et al. 1984). Intestinal samples, taken from a region immediately adjacent to the one collected for the histology, were homogenized (1:10, v/v) in a solution containing aprotinin ($1\mu\text{g ml}^{-1}$ in 100mM potassium phosphate buffer pH 7.4) and centrifuged for 25 min at 10,000rpm at 4°C . Pellets were re-homogenized (1:5, v/v) in 50mM potassium phosphate buffer (pH 6) containing 0.5% hexadecyltrimethyl-ammonium bromide (HTAB) and aprotinin ($1\mu\text{g ml}^{-1}$). Samples were divided into two aliquots, subjected to three cycles of freezing (15min, -80°C) and thawing (20min, 37°C), and then centrifuged for 30min at 12,000rpm at 4°C . An aliquot of the supernatant ($100\mu\text{l}$) was allowed to react with a buffer solution of α -dianisidine (0.167mg ml^{-1}) and 0.0005% H_2O_2 . The rate of change in absorbance was measured with a spectrophotometer at 470nm (Du® 530, Beckman Coulter, Fullerton, CA, USA). One unit of MPO was defined as the quantity of enzyme degrading 1 mmol of peroxide per minute at 25°C . Data were expressed in mU mg^{-1} of wet tissue.

Cytokines and μOR mRNA expression in the ileum

Real-time quantitative RT-PCR—Samples of ileum were homogenized with Power Gen 125 (Fischer scientific, Pittsburgh, PA, USA) and total RNA was extracted using the Absolutely RNA® RT-PCR Miniprep Kit (Stratagene, La Jolla, CA, USA). The quality of RNA was verified by electrophoresis on a 2% agarose gel and the amount of the purified RNA was determined by spectrophotometry (Nanodrop 1000), Thermo Scientific, Waltham, MA, USA). Spectrophotometric analysis of the samples showed absorption ratio (OD) $\text{OD}_{260\text{nm}}/\text{OD}_{280\text{nm}} > 1.9$, indicating excellent purity of the ribonucleic acids. Total RNA ($1\mu\text{g}$) was reverse transcribed using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA, USA).

The expression of μOR , and cytokines TNF- α and IL-10 mRNA was assessed by quantitative RT-PCR using TaqMan Gene Expression Master Mix and Pre-Developed TaqMan Assay (Applied Biosystem, Carlsbad, CA, USA) specific to mouse μOR (Mm01188089), TNF- α (Mm99999068_m1), IL-10 (Mm01288386_m1) and the housekeeping gene β -actin (Mm1205647_g1). The PCR reaction mixture was incubated at 95°C for 10 min and then run for 50 cycles at 95°C for 15 sec and 60°C for 1 min using Stratagene® Mx 3000p™ machine. Expression of β -actin (an endogenous internal control gene) was measured in parallel for every sample and the data obtained for μOR , TNF- α and

IL-10 were normalized to those of the β -actin. Relative quantities (RQ) of mRNA were analyzed using the comparative threshold cycle (C_T) method (Anselmi et al. 2005; Livak and Schmittgen 2001).

Drugs

DAMGO ([D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin), CTAP ([H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂]), *o*-dianisidine, HTAB and aprotinin were obtained from Sigma Aldrich Corporation (St. Louis, MO, USA). Myeloperoxidase was purchased from Calbiochem-EDM Chemicals, (La Jolla, CA, USA).

Statistic analysis

Data are expressed as means $\pm$ SE. Comparison among groups was made using analysis of variance (two-way ANOVA) followed by Bonferroni's post-test. Differences were considered significant when P value was below 0.05. Analysis was performed using Prism 4 program (GraphPad Software, San Diego, CA).

RESULTS

Intestinal histology

Sham operation induced moderate tissue damage characterized by low level of edema and slight epithelial lifting compared to normal mice. There was a significant increase in tissue damage in I/R specimens compared to SO as shown by the significantly increased total score in I/R vs. SO tissue (I/R, 8.43 ± 0.57 ; SO, 3.14 ± 0.91 ; N, 1.25 ± 0.25 ; $p < 0.001$) (Fig.1). Specifically, intestinal I/R induced pronounced neutrophil infiltration within lamina propria and submucosa, severe edema and lifting of the epithelial layer from lamina propria, and muscle thickening (Fig. 2). Treatment with the selective μ OR agonist peptide, DAMGO, significantly reduced histological damage in I/R mice by reducing the level of edema and epithelial lifting, and decreasing the infiltration of inflammatory cells in intestinal tissue (total score I/R treated with DAMGO = 5.60 ± 0.60 , $p < 0.01$) (Figs. 1 and 2). This effect was completely reversed by the co-administration of the μ OR antagonist peptide, CTAP (total score = 9.00 ± 0.45 , $p < 0.001$). CTAP alone did not affect overall histological damage. Treatment with μ OR agonist or antagonist did not affect the histological appearance in the SO and N groups compared to vehicle treated animals (Figs. 1 and 2).

Gastrointestinal transit

There was a significant delay in GI transit both in I/R (geometric centre GC = 3.27 ± 0.27) and in SO (GC = 4.93 ± 0.58) groups compared to N (GC = 7.76 ± 0.5) ($p < 0.001$). The slightly different delay in GI transit in I/R and SO animals was not significant. GI transit in I/R, SO or N was not affected by administration of DAMGO or CTAP (Fig.3). Analysis of the small bowel GC confirmed a significant transit delay in both I/R (GC = 2.17 ± 0.29) and SO (GC = 3.88 ± 0.58) mice compared to normal mice (GC = 6.72 ± 0.50 ; $p < 0.001$), and no significant difference between I/R and SO transit. Similarly, DAMGO did not significantly affect small bowel transit in any of the groups (GC in DAMGO I/R = 3.11 ± 0.43 , in DAMGO SO = 3.50 ± 0.59 and DAMGO N = 6.55 ± 0.37).

The short half life of DAMGO (15 min) (Szeto et al. 2001) raised the question of whether the lack of effect of DAMGO on GI transit observed in our study could be due to the time elapsed between the drug injection and GI transit measurement (about 3 hours). We therefore performed a time course study in normal animals, by injecting DAMGO (0.01 mg/kg s.c.) or saline at 5 and 30 min before the evaluation of the transit (4–5 animals per group). The GC was comparable in each group (GC = 7.08 ± 0.16 and 7.27 ± 0.30 at 5 and 30 min DAMGO injection, respectively, and 7.27 ± 0.10 in saline injected animals). This indicates

that the concentration of DAMGO that reduces the inflammatory response in I/R mice does not affect total GI transit.

Myeloperoxidase activity

MPO activity in tissue homogenates was used to assess the extent of neutrophil recruitment. I/R induced a 5-fold increase in intestinal MPO activity ($p<0.05$), compared to the SO and N animals (Fig. 4). DAMGO treatment resulted in a pronounced and significant reduction of MPO levels in I/R mice compared to saline ($p<0.05$). CTAP treatment completely reversed DAMGO inhibitory effect on MPO activity ($p<0.001$), confirming that it was a receptor-mediated effect. CTAP alone increased MPO activity in I/R compared to saline I/R group, but the differences were not statistically significant. DAMGO and CTAP did not significantly modify MPO activity in SO or N mice, though MPO activity in CTAP treated SO mice was higher compared to saline or DAMGO treated.

Cytokines mRNA expression

TNF- α is a pro-inflammatory cytokine, released from macrophages, monocytes, lymphocytes and other cells, capable of increasing other cytokines release in the inflammatory cascade as well as eliciting leukocyte migration (Markel et al. 2006). The levels of TNF- α mRNA expression quantified by real time PCR were higher in I/R animals compared to SO and N mice ($p<0.05$). Treatment with DAMGO inhibited the production of TNF- α mRNA in I/R mice ($p<0.05$) and this effect was abolished by the co-administration of CTAP (Fig. 5). CTAP alone did not significantly alter the expression of TNF- α mRNA in I/R mice but significantly increased it in SO, suggesting activation of the endogenous opioid system during surgery manipulation. TNF- α mRNA in N mice was not altered by DAMGO or CTAP administration.

IL-10 is a cytokine produced by T-helper2 cells, implicated as an inhibitor of pro-inflammatory cytokine production and of several accessory cell functions of macrophage, T-cells and NK cell lines (Markel et al. 2006). IL-10 mRNA expression was significantly higher in I/R mice ($RQ_folds = 4.57 \pm 0.64$) compared to SO (1.79 ± 0.42 ; $p<0.05$) and N animals (1.14 ± 0.23 ; $p<0.05$). However, the treatment with DAMGO did not alter the expression of this cytokine ($I/R = 5.63 \pm 1.31$, $SO = 0.96 \pm 0.14$, $N = 1.27 \pm 0.2$).

μ OR mRNA expression

The levels of μ OR mRNA in the ileum of I/R, SO and N mice were comparable (RQ_fold 1.58 ± 0.36 in I/R group; 1.53 ± 0.24 in SO group; 1.19 ± 0.33 in N group).

DISCUSSION

This study shows that 45 min intestinal ischemia followed by 5 hours of reperfusion induces mucosal damage with cellular infiltration in the small intestine accompanied by increase in cytokines (TNF- α and IL-10) mRNA expression compared to SO and normal mice. Activation of μ ORs ameliorated I/R-induced intestinal inflammation by reducing neutrophil infiltration and TNF- α mRNA expression, effects that were prevented by μ OR blockade, showing that DAMGO protective effect is receptor-mediated. Since DAMGO does not readily pass the blood brain barrier, this protective effect is likely due to activation of peripheral μ ORs. DAMGO administration did not affect the levels of IL-10 mRNA nor GI transit. These findings support the concept that activation of peripheral μ ORs protects from the acute intestinal inflammation resulting from ischemia/reperfusion through a mechanism involving the pro-inflammatory cytokine, TNF- α .

Intestinal ischemia and reperfusion induce early recruitment of CD4⁺ and CD8⁺ T-cells and release of inflammatory mediators that modulate the recruitment of neutrophils (Linfert et al. 2009; Shigematsu et al. 2002), with the maximal systemic activation occurring within 4–5 hours of reperfusion (Miner et al. 1999). The infiltration of the bowel wall by activated polymorphoneutrophils together with the loss of mucosal integrity due to the interruption of blood supply contribute to the development of acute inflammatory response (Ballabeni et al. 2002; Barocelli et al. 2006), which is likely to be responsible for later complications, including impairment of GI transit, which could derail to a life-threatening condition like the multiple organ dysfunction syndrome. The immune system plays an important role in the development of intestinal inflammation and several signaling molecules, including peptides and their G protein coupled receptors have been proposed as modulators of immune response and inflammation (Gross and Pothoulakis 2007). There is increasing evidence for a regulatory role of the opioid system and in particular the μ ORs on immune processes and inflammation (Chuang et al. 1995; Gaveriaux-Ruff et al. 1998; Madden et al. 1998; Stefano et al. 1996). Indeed, μ OR agonists have been shown to prevent inflammation in a model of experimental colitis suggesting that μ OR agonists might represent potential novel therapeutic approaches for inflammatory bowel diseases (Philippe et al. 2003). In our experimental model, administration of the μ OR selective agonist, DAMGO caused a significant reduction of microscopic mucosal damage and MPO activity, an index of neutrophil infiltration in intestinal tissue, in I/R mice. These effects were receptor mediated since they were prevented by receptor blockade with CTAP, a selective μ OR antagonist. These findings are supportive of a protective effect of μ OR activation in intestinal inflammatory processes induced by ischemia followed by reperfusion and are in agreement with and extend previous observations in experimental colitis (Philippe et al. 2003).

In order to explore the mechanism underlying μ OR protection on I/R-induced inflammation, we evaluated the effect of μ OR activation on the expression of TNF- α and IL-10, two cytokines that modulate inflammatory responses with opposing effects. TNF- α is a pro-inflammatory cytokine, which contributes to both local and distant inflammatory responses in I/R by inducing leukocyte migration and increasing the production of other cytokines and proteolytic enzymes (Markel et al. 2006; Yao et al. 1996). By contrast, IL-10 is a counter regulatory cytokine, which has been regarded as a downregulator of inflammatory response and an inhibitor of pro-inflammatory cytokine production (Markel et al. 2006), even though other studies do not confirm IL-10 protective effect (Nussler et al. 2003). Our findings that DAMGO treatment significantly and markedly reduced TNF- α levels, without affecting IL-10 levels in I/R mice, suggest that suppression of the pro-inflammatory cytokine, TNF- α following μ OR activation could be a mechanism responsible for the diminution of the inflammatory processes generated by I/R, whereas the IL-10 does not appear to be involved in the μ OR protection from I/R induced intestinal inflammation. An interesting observation was the increase in TNF- α mRNA levels induced by μ OR blockade in SO mice compared to saline, which were comparable to the levels in I/R mice. μ OR agonists have been reported to suppress TNF- α mRNA expression in human colonic mucosa in organ culture (Philippe et al. 2006), and endogenous opioids are released following abdominal surgery (Patierno et al. 2004; Patierno et al. 2005) and intestinal ischemic preconditioning (Zhan et al. 2001). Thus, it is tempting to speculate that endogenous opioids released in response to surgery downregulate TNF- α and that μ OR blockade prevents opioid inhibitory effect resulting in increased levels of TNF- α . Since CTAP has been shown to pass the blood brain barrier (Abbruscato et al. 1997), whether its effect on TNF- α in SO involves antagonism at central and/or peripheral μ ORs cannot be established by our results. The lack of effect of μ OR blockade on TNF- α expression in I/R group might be due to the intervention of other regulatory factors due to the ischemia/reperfusion.

μORs are abundantly expressed throughout the body including the brain and peripheral tissue and many of μOR-mediated effects often involve different locations. In the gastrointestinal tract, μORs are located on enteric neurons and inflammatory cells (Sternini et al. 2004). Since DAMGO cannot readily pass the blood brain barrier (Al-Khrasani et al. 2007), the modulatory effect of μOR activation on intestinal inflammation in our model of acute I/R is likely to be predominantly a peripheral effect. μORs protecting from I/R inflammation are probably those expressed in inflammatory cells of the lamina propria, since μOR activation by DAMGO results in reduction of inflammatory cell infiltration, whereas it fails to affect GI transit. However, an involvement of neuronal μORs cannot be excluded at this time. In any case, a participation of central μORs is unlikely and not supported by our data. Damage to enteric neurons has been reported in I/R (Calcina et al. 2005; Rivera et al. 2009), as well as in smooth muscle cells of the gut (Pontell et al. 2011), which are due to inflammatory cell infiltrates extending to the muscle layers and enteric ganglia and are regarded as a triggering factor for the motility disturbances. However, these changes are more prominent at later times of reperfusion, starting between 6–12 hours and becoming more evident at 24 hours, whereas in our study we evaluated the effect of μOR activation at 5 hour-reperfusion when acute inflammation is the most prominent alteration observed. It is reasonable to suggest that reduction of the inflammatory response during the early phase of I/R injury might diminish the risk of developing later complications such as motility impairment. Furthermore, the transit delay we observed in our animals with I/R was comparable to the delay observed in SO mice, which indicates that the surgery itself and the intestinal manipulation not ischemia and reperfusion are responsible for the impaired transit. Enteric μORs are not likely to play a major role in postoperative ileus, since blockade of endogenous opioid release induced by μOR antagonists does not reverse GI transit delay (Patierno et al. 2004). By contrast, peripheral μOR antagonists are effective in reducing the further inhibition of GI transit induced by morphine administered postoperatively. Moreover, peripheral μORs are the main mediators of opioid bowel dysfunction that develops in patients receiving morphine or other opiates for pain control, which is ameliorated by μOR antagonism (Holzer P, 2010). The apparent discrepancy between our and previous studies in rodents showing an increased delay of GI transit following ischemia and reperfusion in comparison to sham operation (Ballabeni et al. 2002; Cattaruzza et al. 2006) can be explained by the different methodology used or different length of reperfusion.

Previous studies showed an upregulation of μOR mRNA in inflammatory bowel disease in humans (Philippe et al. 2006) and in different models of intestinal inflammation in mice (Philippe et al. 2003; Pol et al. 2001). In our study, we did not detect significant differences in μOR mRNA expression in I/R compared to SO and normal mice. This divergency might be due to the different length and/or level of inflammation in our model compared to other experimental models where the inflammatory processes were more chronic (days) than our model (hours). Our data suggest that the protective effect of μOR on I/R-induced inflammation does not require receptor upregulation, but is due to receptor activation and might involve changes occurring downstream of the receptor.

In conclusion, this study provides evidence for a protective role of μORs in intestinal inflammation induced by I/R. This effect is likely to be predominantly due to the activation of peripheral μORs in inflammatory cells and be mediated by the pro-inflammatory cytokine, TNF-α. These findings support the concept that peripheral μORs could be explored as possible targets for novel therapeutic approaches to ameliorate inflammation, the first acute event in intestinal injury induced by ischemia/reperfusion. Reduction of acute inflammation might prevent I/R complications, including motility impairment, which develop at a later stage of reperfusion and are likely due to inflammatory cell infiltrates.

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Figure 1.

Effect of μ OR activation on microscopic damage score in mouse intestine. The level of inflammation in distal ileum was significantly higher in ischemic/reperfused (I/R) compared to sham operated (SO) and normal (N) animals in saline treated group. μ OR activation with DAMGO (0.01 mg/kg s.c.) significantly reduced the inflammatory response in I/R, but did not affect the low levels of inflammation in SO animals. DAMGO effect on the I/R inflammatory response was prevented by pretreatment with the μ OR antagonist, CTAP (0.1 mg/kg s.c.). CTAP alone did not significantly affect the overall I/R-induced inflammatory response. All values are reported as mean $\pm$ SE of 6-- animals. ** p <0.01, *** p <0.001.

Figure 2. Histological damage in mouse intestine. Representative histological sections of mouse ileum from normal [A], and I/R animals treated with saline [B], the selective μ OR agonist, DAMGO (0.01 mg/kg s.c.) alone [C] or in presence of the selective μ OR antagonist, CTAP (0.1 mg/kg s.c.) [D]. I/R induced marked neutrophil infiltration and edema of the submucosa (arrows in B and D), epithelial lifting (arrows in B and D) with mucosal erosion and muscle thickening (arrowheads in B and D). DAMGO markedly reduced histological damage in ischemic/reperfused (I/R) mice [C], effect that was reversed by co-administration of CTAP [D]. Calibration bar: 50 μ m.

Figure 3.

Effect of μ OR activation on gastrointestinal (GI) transit, measured as the geometric center (GC) of the distribution of label dextran orally administered in ischemic/reperfused (I/R), sham-operated (SO) and normal (N) mice. GI motility is significantly delayed in both I/R and SO groups compared to N mice. The slightly different delay in GI transit in I/R compared to SO animals was not significant. GI transit in I/R, SO or N was not affected by administration of selective μ OR agonist DAMGO (0.01 mg/kg s.c.) or selective μ OR antagonist CTAP (0.1 mg/kg s.c.). All values are reported as mean $\pm$ SE of 5–6 animals.

*** $p < 0.001$.

Figure 4.

Effect of μ OR activation on myeloperoxidase (MPO) activity, an index of neutrophil accumulation in intestinal tissue in ischemic/reperfused (I/R), sham-operated (SO) and normal (N) mice. MPO is significantly increased in I/R mice compared to SO and N mice. DAMGO (0.01mg/kg s.c.) significantly reduced the level of MPO activity in I/R, but did not significantly modify MPO levels in SO and N mice. μ OR blockade with CTAP (0.1mg/kg s.c.) completely reversed the DAMGO-induced reduction of MPO activity in I/R mice. CTAP alone did not significantly affect MPO levels in any group compared to saline. All values are reported as mean $\pm$ SE of 8–10 animals. * p <0.05, ** p <0.01.

Figure 5.

Effect of μ OR agonist DAMGO on TNF- α mRNA levels in intestinal tissue of ischemic/reperfused (I/R), sham-operated (SO) and normal (N) mice. TNF- α mRNA levels were significantly higher in the ileum of I/R mice compared to SO and N. DAMGO (0.01 mg/kg s.c.) treatment significantly reduced TNF- α mRNA levels in I/R, effect that was reversed by CTAP (0.1 mg/kg s.c.). DAMGO and DAMGO plus CTAP did not affect TNF- α mRNA expression in SO and N mice. Similarly, TNF- α mRNA levels were comparable in I/R and N ilea of animals treated with CTAP alone, whereas they were significantly increased in CTAP-treated SO mice compared to SO saline-treated animals. All values are reported as mean $\pm$ SE of 8–10 animals. * $p < 0.05$.