Ghrelin is a novel growth hormone (GH)-releasing and orexigenic peptide with anti-inflammatory activities. However, the role of ghrelin in the colonic inflammation is still controversial. The aim of the present study was: 1) to examine the expression of ghrelin and TNF-α mRNA in the inflamed colonic mucosa of patients with ulcerative colitis (UC), 2) to analyze the effect of treatment with exogenous ghrelin on the healing of trinitrobenze sulphonic acid (TNBS)-induced colitis in rats, and 3) to assess the effects of ghrelin treatment on mRNA expression for iNOS and protein expression for COX-2 and PPARγ in intact colonic mucosa and in that with TNBS-induced colitis. Fifteen patients with UC and fifteen healthy controls were enrolled in this study. Expression of ghrelin and TNF-α was assessed by semi-quantitative RT-PCR in the colonic mucosal biopsies from UC patients and healthy controls. In addition, the effect of exogenous ghrelin on healing of TNBS colitis was tested in rats without or with capsaicin-induced functional ablation of sensory nerves. Patients with UC showed a significant upregulation of mRNA for ghrelin and TNF-α in colonic mucosa as compared to that observed in healthy controls. The expression of ghrelin correlated with the grade of inflammation and expression of TNF-α. In rats the exogenous ghrelin administered daily at a dose of 20 µg/kg i.p. significantly accelerated the healing of TNBS colitis and this effect was accompanied by an increase in mRNA expression for iNOS and protein expression for COX-2 in the colonic mucosa. The protein expression for PPARγ, which was down-regulated in rat colonic mucosa after exposure to TNBS as compared to that in intact colonic mucosa, was not significantly influenced by ghrelin treatment. We conclude that 1) patients with UC show an increased mucosal expression of mRNA for ghrelin in the colonic mucosa which could trigger protective response in inflamed colon; and 2) exogenous ghrelin accelerates healing of colonic lesions in animal model of ulcerative colitis via increased release of NO and PGE2 due to an increase in iNOS and COX-2 expression and stimulation of sensory neuropeptides such as CGRP released from sensory afferent endings.

**Keywords:** ghrelin, colitis, nitric oxide, cyclooxygenase-2, sensory nerves, tumor necrosis factor alpha

**INTRODUCTION**

Inflammatory bowel disease (IBD) is a chronic relapsing inflammation of the lower part of the gastrointestinal tract. The main forms of IBD are ulcerative colitis and Crohn’s disease. In the recent decades an unquestionable worldwide increase in the incidence of IBD has been observed. The pathogenesis of IBD remains still not fully understood. It has been postulated that different environmental and genetic factors in combination with the microbial intestinal flora trigger an event which activates intestinal immune (T cells, monocytes, eosinophils, B-cells, neutrophils, etc.) and non-immune systems (epithelial, mesenchymal cells etc.) through secretion of mediators and expression of adhesion molecules, immune and non-immune cells exchange signals resulting in further cell activation and amplification of the production of cytokines, growth factors, nitric oxide, reactive oxygen species, eicosanoids, antibodies etc. culminating in inflammation and tissue damage (1, 2).

Ghrelin is a 28-amino acid peptide mainly produced in the gastric mucosa. It shares several properties of growth hormone-releasing hormone (GHRH) and acts via growth hormone secretagogue receptor (GHS). Interestingly, the presence of an octanoyl group at Ser 3 is essential for its activity (3). Apart from a potent GH-releasing action, ghrelin shows a number of physiological activities such as stimulation of appetite and gastrointestinal motility, cardiovascular effects, etc. (4). Our own studies demonstrated a strong gastroprotective effect of this peptide against acute gastric lesions induced by stress, ethanol or ischemia-reperfusion (5-7).

The fact that the ghrelin receptors are expressed by monocytes/macrophages, B cells and dendritic cells indicates a possible participation of ghrelin in the modulation of colonic inflammatory response in IBD (8). However, the potential role of ghrelin in colitis has not been studied extensively. Recently, Gonzalez-Rey and Delgado (9) demonstrated for the first time the anti-inflammatory action of ghrelin in two different experimental models of colitis (TNBS and DDS-induced colitis) in mice by showing an inhibitory effect of ghrelin on proinflammatory cytokines and Th1-mediated inflammatory response. The strong anti-inflammatory activity of ghrelin was accompanied by a significant reduction in the systemic inflammatory response. Importantly, it was demonstrated that
growth hormone (GH) and IGF-1 do not mediate the effects of ghrelin (9). As a possible important mechanism responsible for the anti-inflammatory activity of ghrelin was postulated the downregulation of nuclear factor kappa B (NFκB) (10). However, the role of ghrelin in colitis remains still controversial since some groups even postulated the proinflammatory actions of ghrelin via increase in NFκB activity (11).

The aim of the present study was: 1) to examine the expression the mRNAs expression of ghrelin and TNF-α in the inflamed colonic mucosa of patients with UC, 2) to determine the effect of exogenous ghrelin on the time course of healing of TNBS-induced colitis in rats, 3) to assess the effects of ghrelin treatment on mRNA expression for inducible NO synthase (iNOS) and protein expression for cyclooxygenase-2 (COX-2) and peroxisome proliferators-activated receptor γ (PPARγ) and; 4) to analyze the involvement of sensory nerves in the anti-inflammatory actions of ghrelin on colonic mucosa in rats with TNBS-induced colitis.

MATERIAL AND METHODS

Expression of ghrelin mRNA in colonic mucosa of patients with UC

The biopsy samples were obtained during colonoscopic investigation from sigma and colon ascenders in 15 patients with active and histologically proved UC and in 15 sex- and age matched healthy controls. The expression of ghrelin was assessed by RT-PCR. Human study protocol was approved by Ethics Committee of the University of Erlangen-Nuremberg, Erlangen, Germany and informed consent was obtained from all subjects before they entered the study.

TNBS-induced colitis and treatment groups of rats

All animal experiments were approved by the Local Institutional Animal Care and Use Committee at the Jagiellonian University College of Medicine, Cracow, Poland and were carried out in accordance with European Union guidelines for the handling and use of laboratory animals. Acute colitis was induced according to published methods (12). Briefly, rats were anesthetized with ketamine and colitis was induced by intrarectal administration of 15 mg of 2,4,6-trinitrobenzene sulfonic acid (TNBS) in 15% ethanol. Following treatment groups (each including 6 animals) received: 1) vehicle (control); 2) TNBS; 3) ghrelin applied in a dose of 20 µg/kg i.p. starting at 3rd day after the induction of the TNBS colitis; 4) L-NNA at a dose 20 mg/kg i.p. to inhibit NO synthase activity starting at 3rd day after induction of TNBS colitis; 5) L-NNA in a dose of 20 mg/kg i.p. co-administered with ghrelin in a dose 20 µg/kg i.p. starting at 3rd day after induction of TNBS colitis.

In the separate experiments, the involvement of sensory neurons in the healing by ghrelin of TNBS-colitis was tested. For this purpose the animals were treated with capsaicin (Sigma) injected s.c. for 3 consecutive days at a respective dose of 25, 50 and 50 mg/kg about 2 weeks before induction of TNBS colitis. All injections of capsaicin were performed under ether anesthesia to counteract the respiratory impairment associated with the injection of this agent. To check the effectiveness of the capsaicin denervation, a drop of 0.1 mg/ml solution of capsaicin was instilled into the eye of each rat and the protective wiping movements were counted as previously described. Control rats received vehicle injections. All animals pretreated with capsaicin showed negative wiping movement test, confirming functional denervation of the capsaicin sensitive nerves. Administration of ghrelin or vehicle saline was applied in rats without or with colitis induced by 3 days earlier by TNBS.

Reverse transcription polymerase chain reaction (RT-PCR)

The human biopsy samples from colon were collected for determination of ghrelin mRNA expression. The colonic samples were excised from intact rats and from those treated with vehicle (control), ghrelin and/or L-NNA with or without induction of TNBS-colitis for the determination of mRNA and protein expression for iNOS using RT-PCR with specific primers. Immediately after the measurement of the area of colonic mucosal damage the mucosal specimens were scraped off on ice using a slide glass and immediately snap frozen in liquid nitrogen and stored at -80°C until analysis. Total RNA was extracted from mucosal samples using a guanidium isothiocyanate/phenol chloroform single step extraction kit from Stratagene (Heidelberg, Germany). Following precipitation, RNA was resuspended in RNase-free TE buffer and its concentration was estimated by absorbance at 260 nm wavelength. Furthermore, the quality of each RNA sample was determined by running the agarose-formaldehyde electrophoresis. RNA samples were stored at -80°C until analysis.

Single stranded cDNA was generated from 5 µg of total cellular RNA using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Stratagene, Heidelberg, Germany) and oligo-(d)T primers (Stratagene, Heidelberg, Germany). Briefly, 5 µg of total RNA was uncoiled by heating (65°C for 5 min) and then reverse transcribed into complementary DNA (cDNA) in a 50 µl reaction mixture that contained 50 U MMLV-RT, 0.3 µg oligo-(dT)-primer, 1 µl RNase Block Ribonuclease Inhibitor (40U/µl), 2 µl of a 100 mM/l mixture of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dTTP) and deoxythymidine triphosphate (dCTP), 10 mM/l Tris-HCl (pH=8.3), 50 mM KCl, 5 mM MgCl2). The resultant cDNA (2 µl) was amplified in a 30 µl reaction volume containing 2 U Taq polymerase, dNTP (200 µM each) (Pharmacia, Germany), 1.5 mM MgCl2, 5 µl 10x polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH=8.3) and specific primers for β-actin, ghrelin, TNFz and iNOS used at final concentration of 1 mM (all reagents from Takara, Shiga, Japan). The mixture was overlaid with 25 µl of mineral oil to prevent evaporation. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) and the incubation and thermal cycling conditions were as followed; denaturation at 94°C for 1 min, annealing at 60°C for 45 sec and extension 72°C for 2 min. The number of cycles was 30 for β-actin, TNF-α and 33 cycles for ghrelin, respectively. The nucleotide sequence of the primers were as follows; human preproghrelin sense: GAG GAT GAA CTG GAA GTC CG; antisense CAT TTA TTC GCC TCC TGA GC; human TNF-α sense: GAG TGA CAA GCC TGT AGC A; antisense GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC T; rat β-actin: sense TTG TTA CCA ACT GGG AGC ATG TTA GG; antisense CAT CCT GAT CTT CAT GGT AGG; rat iNOS: sense: CAG TGG CAA CAT CAG GTC C; antisense: GGT CTC GGA CTC CAA TCT . The primer sequences were based on the sequences of the published cDNAs

Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using 100-bp ladder (Takara, Shiga, Japan) as a standard size marker. The gel was then photographed under UV transillumination. The intensity of PCR products was measured using video image analysis system (Kodak Digital Science) as described earlier. The signal for investigated mRNA was standardized against that of the β-actin mRNA from each sample and the results were expressed as analyzed ghrelin and iNOS mRNA/β-actin mRNA ratio as described earlier (6).
Western blot

For Western blot analysis, proteins were extracted from the same colonic mucosa samples as mentioned above. Approximately 10 µg of total protein extracts was loaded on SDS-polyacrylamide gels and run at 40 mA, followed by transfer onto nitrocellulose membrane (Protran, Schleicher and Schuell, Germany) by electroblotting. Solution of 3% BSA (Sigma Aldrich, Germany) in TBS/Tween-20 buffer (137 mmol NaCl, 20 mmol Tris-HCl, pH 7.4, 0.1% Tween-20) was used to block filters for at least 1 h at room temperature. Specific primary antibody against PPARγ and COX-2 (rabbit polyclonal, dilution 1:200; Santa Cruz, USA) or β-actin (mouse monoclonal, dilution 1:3000; Sigma Aldrich, Germany) was added to the membrane, followed by an anti-rabbit-IgG or anti-mouse-IgG HRP-horseradish peroxidase conjugated secondary antibody (dilution 1:40 000 or 1:20 000) dissolved in 1% non-fat milk in TBS-Tween-20 buffer. Incubation of primary antibody was followed by 3 washes with TBS-Tween-20 buffer for 5 min. Incubation of the secondary antibody was followed by 6 washes for 5 min. Immunocomplexes were detected by the SuperSignal West Pico Chemiluminescent Kit (Pierce, USA). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany).

Statistical analyses

Results are expressed as means ± S.E.M. from 6 rats per group. Statistical significance of difference was determined using analysis of variance (one-way ANOVA). Further statistical analysis for post hoc comparisons was carried out using Bonferroni/Duncan test or Student’s t-test when appropriate. Differences were considered statistically at p<0.05 and this is indicated with an asterisk, cross or slash in the figures.

RESULTS

In control healthy subjects weak but detectable mRNA expressions for ghrelin and TNF-α in colonic biopsies were observed. In contrast, patients with UC showed significantly higher mRNA expression of ghrelin and TNF-α in colonic mucosa. The expression of ghrelin and TNF-α was significantly higher in the sigma than in the colon ascendens (Fig. 1AB).

As shown in the Fig. 2, patients with left sided colitis showed an increased mRNA expression of ghrelin and TNF-α only in the sigma, but not in the colon ascendens. In contrast, patients with pancolitis showed an increased mRNA expression for ghrelin and TNF-α both in sigma and colon ascendens.

The intrarectal administration of TNBS in rats induced severe mucosal injury characterized by necrosis of the epithelium and focal ulcerations of the mucosa. The colonic mucosal blood flow was reduced by about 42%. The treatment with ghrelin was associated with a significant reduction in the mean lesion area in the colon (by 50%) and significant increase in colonic blood flow as compared to that observed in vehicle-treated animals with TNBS colitis. As shown in the Fig. 3, the co-administration of NO synthase blocker, L-NNA to rats with TNBS colitis, caused a significant aggravation in the mean lesion area and a profound reduction in the colonic blood flow as compared to those in vehicle-treated TNBS colitis. The administration of L-NNA before application of ghrelin in TNBS-induced colitis rats completely abolished the healing effects of ghrelin on colonic mucosa and also eliminated the vasodilator effect of this peptide (Fig. 3).

To study the role of sensory nerves in the healing effects of ghrelin on the colonic mucosa, in separate experiments TNBS was administered to rats with capsaicin-ablated sensory nerves. The inactivation of sensory nerves caused a significant increase in mean

![Fig. 1. Densitometric analysis of mRNA expression of ghrelin in colonic mucosa (Colon ascendens and sigma/rectum) of patients with UC and healthy controls, presented as a ratio of ghrelin mRNA over GAPDH mRNA (upper panel-A). Densitometric analysis of mRNA expression of TNF-α in colonic mucosa (Colon ascendens and sigma/rectum) of patients with UC and healthy controls, presented as a ratio of ghrelin mRNA over GAPDH mRNA (lower panel-B). Asterisk indicates significant increase of this ratio as compared to that observed in colonic mucosa in healthy controls. Cross indicates significant increase above the value observed in colon ascendens.](image)

![Fig. 2. Representative RT-PCR showing the expression of mRNAs for ghrelin, TNF-α and GAPDH (house-keeping gene) in the mucosa of colon ascendens (1) and in sigma (2) of healthy controls and in patients with left-sided ulcerative colitis (UC 1) and patients with pancolitis (UC 2).](image)
lesion area and a significant decrease in colonic mucosal blood flow compared to those in rats without capsaicin deactivation (Fig. 4). In rats with inactivated sensory nerves by capsaicin the healing and vasodilatory effects of ghrelin in colon mucosa were significantly smaller when compared to those observed with ghrelin in rats without capsaicin deactivation (Fig. 4).

At the mRNA level, the intrarectal administration of TNBS resulted in the increase in ghrelin mRNA expression starting from day 3 and reaching its maximum at day 7th after induction of colitis (Fig. 5). At days 10 and 14 after induction of colitis with TNBS, the ghrelin expression decreased to the level observed in the control rats without TNBS colitis but instead administered with saline (Fig. 5).

**Fig. 3.** Mean area (mm²) of colonic lesions induced by TNBS and accompanying changes in the colonic blood flow measured in intact rats treated with vehicle (saline) or ghrelin at a dose of 20 µg/kg i.p. with or without pre-treatment with L-NNA (20 mg/kg i.p.). Mean ± SEM of 6 rats per group. Asterisk (*) indicates significant changes compared to vehicle treated rats with TNBS colitis; cross (+) indicates significant changes compared to the vehicle-treated TNBS rats and the slash (#) indicates significant change compared to that observed in ghrelin-treated TNBS rats.

**Fig. 4.** Mean area (mm²) of colonic lesions induced by TNBS and accompanying changes in the colonic blood flow measured in rats with or without denervation of sensory nerves with capsaicin and treated with vehicle (control) or ghrelin at a dose of 20 µg/kg i.p. Mean ± SEM of 6 rats per group. Asterisk (*) indicates significant change observed in ghrelin-treated rats compared to that in vehicle-treated controls; cross (+) indicates significant change compared to vehicle (saline)-treated TNBS colitis rats; asterisk with cross (*+) indicate significant change as compared to ghrelin-treated rats without capsaicin denervation.

**Fig. 5.** The mRNA expression for ghrelin in colonic mucosa of intact rats and in colonic mucosa of rats at days 3, 7, 10 and 14 after induction of TNBS colitis. Means ± SEM of 6 rats per group. Asterisk (*) indicates a significant change compared to intact colonic mucosa.

**Fig. 6.** The mRNA expression for iNOS in the colonic mucosa of intact rats treated with vehicle and in colonic mucosa of rats with TNBS colitis after 14 days upon induction of the colitis without or with ghrelin treatment at 14 days after induction of TNBS colitis. Mean ± SEM of 6 rats per group. Asterisk (*) indicates a significant change compared to intact colonic mucosa and asterisk with cross (*+) indicate significant change compared to that observed in rats with TNBS colitis without ghrelin treatment.
The iNOS mRNA expression measured by RT-PCR was almost negligible in vehicle-treated control (intact colon) rats. The induction of TNBS colitis was associated with a small but significant increase in the mRNA expression for iNOS observed at 14th day upon the induction of colitis by TNBS. The treatment with ghrelin of rats with TNBS-induced colitis resulted in a significant and strong upregulation of mRNA for iNOS (Fig. 6).

At protein level, the expression of COX-2 in intact colon mucosa was detected as a very weak signal. In contrast, the PPARγ expression was detected as a strong signal in the intact colonic mucosa. The induction of colitis by TNBS was associated with a significant increase in COX-2 expression observed at day 14 upon this induction and this was accompanied by a significant decrease in PPARγ expression (Fig. 7). Treatment with ghrelin the expression significantly increased expression of COX-2 protein above that observed in rats with TNBS colitis alone without ghrelin treatment, while the PPARγ expression remained at the same low level as in rats with TNBS colitis without ghrelin treatment (Fig. 7).

**DISCUSSION**

The results of this study demonstrate that ghrelin may be implicated in the colonic inflammation as an anti-inflammatory and vasodilatory substance, which could be possibly triggered by the damage to colonic mucosa. This notion is supported by the fact that the mRNA expression of ghrelin in the colonic mucosa of patients with UC was significantly upregulated in the area of inflamed colonic mucosa. The exact role of ghrelin in the colonic inflammation in humans remains unclear but results of our study are in keeping with the observation of Karmiris et al. (13) showing that the serum levels of ghrelin are significantly increased in patients with IBD.

To elucidate the role of ghrelin in the modulation of colitis, the experimental colitis with TNBS was induced in rats. In this model that mimics many features of human colitis, 2,4,6-trinitrobenzene sulfonic acid (TNBS) is delivered intrarectally and the inflammation results from a covalent binding of the haptenizing agent to autologous host proteins with subsequent stimulation of a delayed-type hypersensitivity to TNBS-modified self antigens (14). Using such rats with colitis we demonstrated a strong anti-inflammatory and vasodilatory effect of exogenous ghrelin, which reduced the mean lesion area in colon by about 50% and increased significantly the colonic mucosal blood flow. The amelioration of colonic mucosal injury by ghrelin was accompanied by a significant upregulation of inducible NO synthase indicating an involvement of NO in the healing effect of ghrelin on experimental colitis. In addition, the concurrent treatment with of NO-synthase inhibitor (L-NNA) completely abolished the anti-inflammatory effects of ghrelin against TNBS-induced mucosal injury. These results are in keeping with the previous reports showing the contribution of endogenous NO in the protective effect of ghrelin against acute gastric mucosal injury (6, 7, 15). However, the exact role of iNOS in colonic inflammation remains still controversial and is the subject of intense investigation. A substantial number of the experimental studies demonstrated an improvement of colitis with iNOS inhibition (16). There are also several reports showing an exacerbation of colitis with application of iNOS inhibitors (17). It is known that iNOS expression is regulated by several pro-inflammatory cytokines and it functions to generate high levels of NO via oxidative metabolism of L-arginine. NO seems to have a dual role in colitis leading to opposite results in IBD models. On one hand, NO leads to vasodilation, host defense and inhibition of neutrophil activation and platelet aggregation. On the other hand, NO is a highly reactive free radical and may rapidly react with active oxygen to generate peroxynitrate, which causes severe detrimental effects on colonic mucosa (18).
The present study demonstrated a significant downregulation of peroxisome proliferators-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily of transcription factors. Previous studies demonstrated that PPARγ plays a crucial role in the control of intestinal inflammation and its resolution (19). Numerous studies demonstrated that the activation of PPARγ by specific ligands causes an amelioration of colitis (20, 21). The anti-inflammatory effects of PPARγ agonists were partly mediated by inhibition of NFκB and p38 MAPK signaling pathways (22). In the present study we demonstrated that the healing effects of ghrelin treatment have no significant influence on PPARγ expression that remained at decreased level as compared to rats with TNBS colitis without ghrelin treatment.

An important finding of the present study was the increase in COX-2 protein expression in colonic mucosa in rats with TNBS-induced colitis treated with ghrelin. This fact supports the role of COX-2 derived prostaglandins in ghrelin-mediated healing of TNBS-induced colitis. In agreement with our present observation, the exacerbation of colitis by COX-2 specific inhibitors was currently documented (23). In addition, the recent study by Tsubouchi et al (24) revealed a significant increase in mucosal PGE2 generation in the experimental colitis that was attenuated by specific COX-2 inhibitor such as rofecoxib but not COX-1 specific inhibitor such as SC-560. All these data together indicate an involvement of COX-2 derived prostaglandins in healing of colonic lesions by ghrelin and these results are corroborative with our previous findings that ghrelin attenuated gastric damage induced in acute model of ischemia-reperfusion in rats (5-7).

Of physiologic relevance is the observation that deactivation of sensory nerves by capsaicin was associated with aggravation of TNBS-induced colitis and the partial attenuation of protective effect of ghrelin. This observation suggests an importance of integrity of sensory nerves in the healing effects of ghrelin as shown in our previous studies in the different models of acute gastric injury (6, 7). Our results are in agreement with the earlier studies showing an aggravation of colonic inflammation by sensory denervation with capsaicin and an improvement in healing of colonic injury by neuroepithelium CGRP released from these nerves (25, 26).

Taken together, our study demonstrates that ghrelin expression is upregulated in colonic mucosa of patients with UC and that treatment with this peptide exerts a beneficial anti-inflammatory effect on experimental colitis, resulting in acceleration of colonic lesions healing. The amelioration of experimental colitis by ghrelin is, at least partly, due to the increased release of NO, stimulation of COX-2-derived PGE2 and depends on the integrity of sensory nerves. Ghrelin does not influence the PPARγ expression considered as protective factor in IBD (17) that is according to our present study significantly decreased in TNBS-induced colitis.

Conflict of interest statement: None declared.

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Received: August 29, 2008
Accepted: April 30, 2009

Author’s address: Professor Peter Konturek, M.D., First
Department of Medicine, University Erlangen-Nuremberg,
Ulmenweg 18, 91054 Erlangen, Germany.