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Nutrition physiology / Full Paper

The effects of exogenous ghrelin on dextran sodium sulfate-induced colitis in lean and obese mice

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Abstract

Ghrelin is a peptide hormone possessing a variety of physiological and pharmacological actions. This study aims to investigate the anti-inflammatory effects of exogenous ghrelin on chemically induced colitis in genetically predisposed lean (TSNO) and obese (TSOD) mice after different schedule of administration. To induce colitis, animals were given drinking water containing 2% dextran sodium sulfate (DSS) for 5 days. The TSOD and TSNO mice received daily intraperitoneal injections with saline (100 μl/day) or ghrelin (70 nmol/kg/day) for 5 days simultaneously or after DSS treatment. The severity of colitis was assessed by measuring body weight, colon length, histological analysis, plasma tumor necrosis factor α (TNFα) concentration and expression of pro-inflammatory cytokines in the colonic mucosa. At day 10, ghrelin administered after the DSS treatment slightly enhanced colonic inflammation in TSNO mice. On the other hand, ghrelin administration resulted in the partial improvement of colonic inflammation in TSOD mice. Furthermore, ghrelin administered simultaneously with DSS treatment might have slightly ameliorated some inflammation, as indicated by values compared with the after-treatment mice. Our findings suggested that the effects of ghrelin on chemically induced colitis were different between lean and obese mice, and depend on the timing of ghrelin treatment. Therefore, we should consider these points when using ghrelin as an anti-inflammatory agent in inflammation models.

Introduction

Inflammatory bowel disease (IBD) is a chronic disease that causes unexplained inflammation of the digestive tract, mainly consisting of ulcerative colitis (UC) and Crohn's disease [21]. UC forms erosions and ulcers in the large intestine, and shows characteristic clinical symptoms such as diarrhea, bloody stools, abdominal pain, and weight loss [21]. UC has been rapidly increasing in Japan since 1970, and is designated as an intractable disease since its etiology is still unclear and an effective treatment strategy has not been established. Although the pathogenesis is unknown, it has been suggested that persistent inflammation and an antigen causing hyperimmune response in the intesti-

nal tract via immunocompetent cells, both contribute to the development of UC [1]. In addition, there is mounting evidence that both genetic and environmental factors are also intricately involved [20]. IBD patients also have a high risk of colon cancer [7], because chronic inflammation is one of the major causes of carcinogenesis.

Ghrelin, a peptide hormone composed of 28 amino acids with *O*-n-octanoyl acid modification at the serine 3 position, is mainly secreted by the endocrine cells of both the stomach and hypothalamus, and exhibits a variety of physiological functions, such as modulation of food intake and energy homeostasis [12]. Furthermore, there is accumulating evidence suggesting that ghrelin has significant anti-inflammatory activities in various tissues [24, 25]. Previous studies have reported that ghrelin shows protective and healing effects in the gastrointestinal tract. Pretreatment with ghrelin reduces gastric alendronate [9], as well as accelerates the healing of gastric ulcers induced by acetic acid [3]. Ghrelin reduces intestinal inflammation in mouse and rat models of IBD [6, 13, 19]. On the other hand, some investigators reported the pro-inflammatory effect of exogenous ghrelin [5, 22]. It has been suggested that the anti-inflammatory effect of ghrelin is demonstrated by different results depending on the specific experimental conditions used such as its dose or timing of administration.

Obesity causes major adverse health outcomes such as type 2 diabetes, cardiovascular diseases, and dyslipidemia. Both obesity and UC are known as chronic inflammatory diseases, since they also cause increases in immune cells and pro-inflammatory cytokines. However, the relationship between obesity and the development of colitis has not been fully understood. In obese mice, DSS-induced colitis worsened when compared with lean mice. In these cases, experimental colitis and obesity were aggravating factors for each disease [26]. In contrast, in obese rats treated with trinitrobenzene sulfonic acid, both colitis and colonic damage decreased when compared with lean mice [8]. Therefore, the relationship between obesity and colitis remains unclear. In order to clarify the relationship of obesity and colitis, and the anti-inflammatory role of ghrelin on colitis, further studies focusing are really needed.

There have been few reports about the efficacy of ghrelin on colitis developed in obese animals. Since ghrelin has a fat accumulation effect [28], it is interesting to investigate the anti-inflammatory effects of ghrelin on colitis in obese individuals. We, therefore, in the current study, attempted to elucidate whether exogenous ghrelin exerts any anti-inflammatory effects on dextran sodium sulfate (DSS)-induced colitis in obese (TSOD) and lean (TSNO) mice, and if it made a difference depending on the time when ghrelin was administered.

Materials and Methods

Animals

Healthy 8-week-old male TSNO mice (mean body weight ± SD: 29.7 ± 1.7 g) and TSOD mice (mean body weight ± SD: 42.5 ± 2.7 g) were obtained from Sankyo Lab Service Corporation, Inc. (Tokyo, Japan). They were housed in a specific pathogen-free facility at controlled temperature (25°C) with alternating 12:12-h light-dark cycles. Standard mouse chow pellets (CE-2, CLEA Japan, Inc., Tokyo) and tap water were supplied *ad libitum*. This study was approved by the Animal Ethics Committee of Rakuno University (VH25A9).

Induction of colitis and administration of ghrelin

Colitis was induced by adding 2% (wt/vol) DSS (molecular mass 36,000–50,000 Da, MP Biomedicals, Solon, OH, USA) to the drinking water for 5 days *ad libitum*. After one week of acclimatization, 9-week-old male mice were randomly divided into four groups (n = 6 per group, Fig. 1). Group 1 (vehicle control) was administered saline (100 μl) by intraperitoneal (i.p.) injection once per day from day 5 to day 10. Group 2 (DSS alone) was treated with 2% (wt/vol) DSS in their drinking water from day 0 to day 5 to induce colitis and then were administered saline by the same method used for Group 1. Group 3 (DSS + ghrelin) was administered 2% DSS and simultaneously treated with daily i.p. injections of ghrelin (Peptide institute, Inc., Osaka, Japan) at a dose of 70 nmol/kg body weight from day 0 to day 5. Group 4 (DSS → ghrelin) was given 2% DSS in their drinking water from day 0 to day 5, and then was given daily i.p. injections of ghrelin from day 5 to day 10. Ghrelin and saline i.p. injections were conducted at 10:30 AM on each administration day. Mice were monitored daily for reduction in body weight, diarrhea, and bloody stools. After an overnight fasting (for about 12 h), all animals were anesthetized with pentobarbital (40mg/kg, i.p.) in the morning on day 10 and blood was collected from the caudal vena cava. After sacrifice, tissues (colon, liver, and spleen) were removed for the experimental analysis.

Histopathological grading of colitis

After measurement of the colon length, the large bowels were fixed in 10% (wt/vol) buffered formalin for histopathological examination. Inflammation in the large bowel was scored on the hematoxylin and eosin (H&E)-stained sections. Large intestinal inflammation was graded according to the morphological criteria described by Cooper et al. [4]: grade 0, normal appearance; grade 1, shortening and loss of the basal 1/3 if the actual crypts with mild inflammation in the mucosa; grade 2, loss of the basal 2/3 of the crypts with moderate inflammation in the mucosa; grade 3, loss of all of the crypts with severe inflammation in the mucosa and submucosa, while retaining the surface epithelium; and grade 4, presence of mucosal ulcer with severe inflammation (infiltration of neutrophils, lymphocytes, and plasma cells) in the mucosa, submucosa, muscularis propria, and/ or submucosa. The scoring was performed on the entire colon and expressed as a mean average score/mouse.

Measurement of plasma tumor necrosis factor α (TNFα) concentrations

After an overnight fast, blood was collected in EDTA-coated blood collection tubes, centrifuged at 1,500 × *g* for 15 minutes at 4°C, and the supernatants collected. TNFα levels were determined using commercial assay kits, Mouse TNFα Quantikaine ELISA kits (Cosmo Bio Co., ltd., Tokyo, Japan), according to the manufacturer's instructions.

RNA extraction and quantification by real-time PCR

Total RNA was extracted from the colonic mucosa using the ISOGEN reagent (Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's protocol. cDNA was then synthesized from total RNA using ReverTra Ace reverse transcriptase (TOYOBO Co., Osaka, Japan). Real-time PCR analysis of individual cDNA was performed using primers for mouse tumor necrosis factor α (TNFα, sense: TATGGCCCAGACCCTCACA, antisense: GGAGTAGACAAGGTACAACCCATC), cyclooxygenase2 (COX2, sense: CTGGAACATGGACTCACTCAGTTTG, antisense: AGGCCTTTGCCACTGCTTGTA), and interleukin 1β (IL-1β, sense: TCCAGGATGAGGACATGAGCAC, antisense: GAACGTCACACACCAGCAGGTTA), which were purchased from Takara Bio Inc. (Shiga, Japan). The 18S-rRNA gene was used as an endogenous reference and it was amplified with a LightCycler (Light Cycler System ®1.5, Roche Diagnostics, Basel Schweiz) using SYBR® Premix DimerEraser™ (Takara Bio Inc.), THUNDERBIRD® SYBR qPCR Mix (TOYOBO Co.), and specific primers (sense: GCAATTATTCCCCATGAACG, antisense: GGCCTCACTAAACCATCCAA). PCR cycling conditions were 95℃ for 30 sec, followed by 50 cycles of 95℃ for 5 sec, 55℃ for 10 sec (in case of THUNDERBIRD) or 30 sec (in case of DimerEraser) and 72℃ for 30 sec. The expression levels of the target gene were calculated by the relative standard curve method and were determined as ratios relative to 18S-rRNA expression. Data are presented as fold-change values of treated samples relative to that of Group 1.

Statistical analysis

All of the data in this study were presented as mean ± SEM. The InStat Version 3.05 (Graph Pad Softwares, Inc., San Diego, USA) was used for statistical analyses. Statistical significance was determined by one-way ANOVA, followed by a Tukey-Kramer Multiple Comparisons test or a two-tailed Student's t-test. Values of $P < 0.05$ were considered statistically significant.

Results

General observation

During the study, oral administration of DSS for five days induced acute colitis characterized by body weight loss in the TSNO (lean) mice, starting on day 6 (Fig. 2a), and Group 2 (DSS alone) had a significant percent change in body weight compared with Group 1 starting on day 7 (Fig. 2b). In TSNO mice, administration with ghrelin further reduced the body weight when compared to Group 2 (DSS alone), starting on day 5, when the difference in the reduction in body weight became statistically significant between the two groups and continued to be observed from day 5 to day 8 (Group 2 vs. Group 4) and from day 7 to day 9 (Group 2 vs. Group 3), respectively ($P<0.05$, Fig. 2b). At day 9, the changes in body weight of both Groups 3 and 4 finally decreased, when compared to day 0, to 82% and 85%, respectively. On the other hand, body weights in the TSOD mice were comparable among the groups during the study, although Group 2 had a significantly reduction in body weight from day 7 (Fig. 2). Liver weights on day 10 were comparable among the treatment groups of the TSNO and TSOD mice, although the values of TSOD mice were greater than those of the TSNO mice (Table 1). On day 10, treatment of DSS, with or without ghrelin, increased the spleen weights in both genotypes (Table 1). Shortening of the colon length was observed in both TSNO and

TSOD mice treated with DSS (Group 2). In TSOD mice, DSS treatment significantly shortened the colon length ($P < 0.05$), while ghrelin treatment (Groups 3 and 4) slightly elongated the colon length (Table 1). In both genotypes, simultaneous administration of DSS with ghrelin (Group 3) tended to reduce the shortening of colon length.

Ghrelin did not affect colon damage and acute colitis induced by DSS

The histopathological alterations in the colons were assessed on H&E-stained sections (Figure 3a), and the inflammation scores are shown in Figures 3b. In the control mice (Group 1), the colon presented normal morphology of crypts, abundant goblet cells, and a small number of lamina propria mononuclear cells. In both genotypes, there were no signs of mucosal thickening and the complete absence of ulcerations was noted. However, in Group 2, DSS induced acute colitis with ulceration. Histological analysis revealed severe epithelial damage with extensive cellular infiltration into the submucosa, lamina propria, and colonic mucosa; and depletion of goblet cells, mucosa thickening, and complete destruction of the architecture. These histological changes resulted in a high inflammation score were observed in both genotypes (Fig. 3b). The inflammation scores of Groups 3 and 4 in the TSOD mice were lower than the corresponding scores in the TSNO mice. In TSOD mice, treatment with ghrelin both reduced the infiltration of inflammatory cells into the submucosa and lamina propria and improved the loss of colonic crypts and epithelial cell necrosis. On the other hand, treatment with ghrelin did not improve these histological symptoms in TSNO mice.

The timing of ghrelin administration resulted in different plasma TNFα concentration

Figure 4 shows the plasma TNFα concentration in all groups of TSNO and TSOD mice. Administration of DSS alone (Group 2) increased serum TNFα level when compared to that in the control (Group 1) in both genotypes. Administration of ghrelin combined with DSS (Group 3) tended to reduce serum TNFα concentration compared with that in the DSS alone group (Group 2) in both genotypes. On the other hand, treatment with ghrelin after DSS exposure (Group 4) did not affect the level in TSNO mice, but increased TNFα concentration in TSOD mice. In Group 4, the TNFα level in TSOD mice was significantly higher than that in TSNO mice ($P < 0.05$).

Ghrelin affected the mRNA levels of pro-inflammatory cytokines in the colonic mucosa

Figure 5 presents the qRT-PCR-measured expression levels of pro-inflammatory cytokines that include TNFα (Figure 5a), COX-2 (Figure 5b), and IL-1β (Figure 5c) in the colonic mucosa of the TSNO and TSOD mice. In the DSS alone group (Group 2), the expression of all three cytokines was higher than that in the control group (Group 1) in both genotypes. The expression of all of three cytokines in TSNO mice was higher than that in the TSOD mice in Group 2 (2.4-fold in TNFα, 9.1-fold in COX-2, and 4.1-fold in IL-1b). Ghrelin treatment together with DSS (Group 3) tended to decrease the expression of TNFα, COX-2and IL-1β mRNAs in the TSNO mice. But, this treatment elevated the expression of both COX-2 and IL-1β in the TSOD mice. In contrast, ghrelin administration after DSS treatment (Group 4) elevated the expression of all three cytokines measured in both genotypes compared with that in Group 2.

Discussion

The involvement of obesity and the role of ghrelin in IBD have not been elucidated up to the present. In this study, we examined the differences of the anti-inflammatory effects of ghrelin (simultaneous treatment and post treatment) in both lean and obese mice. To accomplish the objectives, we used genetically predisposed lean (TSNO) and obese (TSOD) mice to compare the effects of ghrelin on DSS-induced colitis.

The results of this study showed that TSNO mice were induced colitis stronger than TSOD mice as evident from body weight change (Fig. 2) and expression of pro-inflammatory cytokines (Fig. 5). Pro-inflammatory cytokines are increased in colitis. Generally, these cytokines are increased in obesity [2, 27]. Therefore, it is considered that obesity directly contributes to systemic inflammation. Several studies have reported a relationship between obesity and the levels of inflammatory proteins such as TNFα, IL-1β, IL-6, and others [2, 27]. Furthermore, other studies have suggested that adipokines, secreted by adipose tissue, are closely associated with UC [10, 14, 18, 23, 29]. Therefore, it was hypothesized that TSOD mice would have more serious DSS-induced colitis compared with TSNO mice, but unexpected results were obtained in this study (i.e., TSNO mice had more serious DSS-induced colitis). The causal relationship between obesity and colitis remains unclear till now. Teixeira et al. [26] reported that obese mice had both prolonged and aggravated DSS-induced inflammatory

manifestations of UC rather than lean mice, whereas Hyland et al. [8] reported that trinitrobenzene sulfonic acid (TNBS)-induced colitis was significantly reduced in diet-induced obese rats compared with diet-resistant rats. Further research will be therefore required to gain a better understanding about the complex relationship between obesity and colitis.

There are some reports supporting the anti-inflammatory effects of exogenous ghrelin on colitis in lean animals [6, 15, 16]. Although many researchers reported that ghrelin has an anti-inflammatory effect in colitis, in this study, a clear anti-inflammatory effect of exogenous ghrelin (70 nmol/kg, bw) was not observed in the TSNO mice. De Smet et al. [5] reported that post-treatment with ghrelin (100 nmol/kg, twice daily) for 5 or 10 days enhanced the clinical disease activity and promoted infiltration of neutrophils and colonic IL-1β levels in DSS-induced colitis. Siegl et al. [22] reported that ghrelin (0.5 μg/g: 148.4 nmol/kg, bw, twice a day, i.p.) conferred protective effects during the early phase of sepsis, but during the later phase, ghrelin administration deteriorated both immune response and outcome in a mouse peritonitis model. Although many differences exist among previous ghrelin studies regarding its dose and treatment schedule, it was thought that post-treatment with high-dose ghrelin (more than 100 nmol/kg, bw) for long periods, might aggravate colitis. Therefore, our results might have been different if we tried to decrease both the dose of ghrelin and the duration of administration. These modifications might have enabled ghrelin to ameliorate the colitis more clearly in the lean TSNO mice.

Some of the differences were observed in the results from TNSO (lean) and TSOD (obese) mice. Ghrelin administration did not affect body weight change and ameliorated the inflammation score in TNOD mice but not in TNSO mice. In addition, in this study, the result of plasma TNFα concentration did not correlate with that of TNFα mRNA expression in the colonic mucosa. In fact, TSOD mice that were treated with exogenous ghrelin after DSS administration (Group 4) had increased plasma TNFα concentrations, whereas similar effect was not observed in TSNO mice. On the other hand, the same treatment increased the expression of TNFα mRNA in the colonic mucosa of TSNO mice, but did not affect that of TSOD mice. Although it remains a matter of speculation, it was thought that exogenous ghrelin treatment after DSS administration exacerbated the pre-existing colitis in TSNO mice, but did not affect plasma TNFα concentration. In contrast, the increase in plasma TNFα concentration of Group 4 in TSOD mice could be due to the secretion of adipokines and TNFα from adipocytes following administration of ghrelin immediately before sampling, but was not colitis related. There are relatively few reports investigating the effects of exogenous ghrelin on obesity in animals. Using a peritonitis mouse model, Siegl et al. [22] demonstrated that ghrelin treatment improved survival, ameliorated hypothermia and increased hyperleptinemia in obese mice compared to that in lean controls. This is partially in accordance with our results. Because it has been unclear whether ghrelin exerts some beneficial effects in obese animals, the modifying effects of ghrelin on some pathological condition, such as colitis in obese animals, warrant further study.

Although the present study could not elucidate the differences in the anti-inflammatory effects of exogenous ghrelin between lean and obese mice, at least it is clarified that ghrelin-induced effects are different in the administration timing of ghrelin or genotypes. Interestingly, it has been reported that exogenous ghrelin (3 nmol/day, i.p.), which was administered during the DSS-treatment periods, significantly suppressed tumor incidence in the inflammation-associated colon carcinogenesis model [11]. Additionally, ghrelin has been expected to be a target for treating cachexia because of its multiple actions, including increasing food intake, decreasing energy expenditure and inflammation, increasing growth hormone, and direct anabolic effect on the skeletal muscles and adipose tissue [17]. However, in the present study, some of the data showed that ghrelin administration tended to aggravate DSS-induced colitis in both lean and obese mice. Therefore, it is necessary to consider carefully the potentially high risk of aggravating the pathological condition depending on the administration timing or dosage of ghrelin and the genotype.

The current study is the first to compare and investigate the chemopreventive effect of ghrelin on colitis using both lean and obese mouse models and at different administration schedule. The findings in the present study suggests that it is necessary to consider both individual's body mass and administration timing when ghrelin-induced anti-inflammatory effects are assessed in colitis.

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和文摘要

肥満と非肥満状態のマウスに誘起した大腸炎に対するグレリンの抗炎症効果に差があるか否か明らかにすることを目的として、肥満モデルマウス及びその野生型マウスを用いて dextran sodium sulfate （DSS）誘発大腸炎に対するグレリン投与（同時処置と後処置）の影響を検討した。野生型(TSNO)及び肥満型(TSOD)マウス各 24 匹を 1 群 6 匹とし、無処置群（1 群）、DSS 単独投与群（2 群）、DSS 及びグレリン同時投与群（3 群）、DSS 投与後グレリン処置群（4 群）を設定した。炎症の指標として、体重、大腸長、ELISA による血中 TNFα濃度解析、大腸の HE 染色による炎症スコア解析及び摘出した大腸の炎症性サイトカイン mRNA 発現の解析を行なった。HE 染色による炎症像及び炎症性サイトカイン mRNA の発現の結果から TSOD マウスと比べ TSNO マウスの方で炎症が顕著だった。グレリン投与（70 nmol/kg BW）に対しては、TSNO マウスでは、いずれの処置でも体重減少が見られたが、炎症スコア、血中 TNFα濃度には変化が見られなかった。また、炎症性サイトカインの発現は、同時処置で若干改善傾向が見られたが、後処置により悪化する傾向が観察された。TSOD マウスでは、体重に変化は見られなかったが、炎症スコアの若干の改善が見られた。しかし、血中 TNFαおよび炎症性サイトカインの発現に関して、改善傾向は見られなかった。以上、グレリン 70 nmol/kg の腹腔内投与では明確な抗炎症効果が得られないこと、グレリンの大腸炎に対する抗炎症効果は、適用スケジュールと肥満の程度で差が出る可能性が本実験により示された。すなわち、グレリンの抗炎症効果を観察する際には、投与量や投与スケジュール、動物の状態を慎重に考慮する必要性があることが示唆された。

キーワード：潰瘍性大腸炎、肥満、グレリン、マウス

Table 1　Body, liver, spleen weight and colon length of mice at day 10

Group		Body weight (g)	Liver weight (g)	Spleen weight (g)	Colon length (cm)
TSNO	G1	33.7±0.45	1.3±0.05	0.07±0.003	7.3±0.28
	G2	29.7±1.11	1.2±0.15	0.19±0.049	6.1±0.33
	G3	26.7±2.63[a]	1.2±0.11	0.22±0.039	7.0±0.32
	G4	28.1±1.64	1.3±0.07	0.24±0.035	6.7±0.25
TSOD	G1	47.1±0.85	1.9±0.10	0.08±0.006	8.6±0.30
	G2	45.5±1.25	1.7±0.08	0.24±0.040	7.3±0.34[c]
	G3	44.5±1.10	1.7±0.06	0.22±0.047	7.8±0.21
	G4	45.6±0.54	1.8±0.07	0.28±0.051[b]	7.4±0.30

Data are mean±SEM of 4 to 6 animals per group.
[a]$P<0.05$ vs. G1 in the TSNO, [b]$P<0.01$ vs. G1 in the TSOD, [c]$P<0.05$ vs G1 in the TSOD

Fig. 1

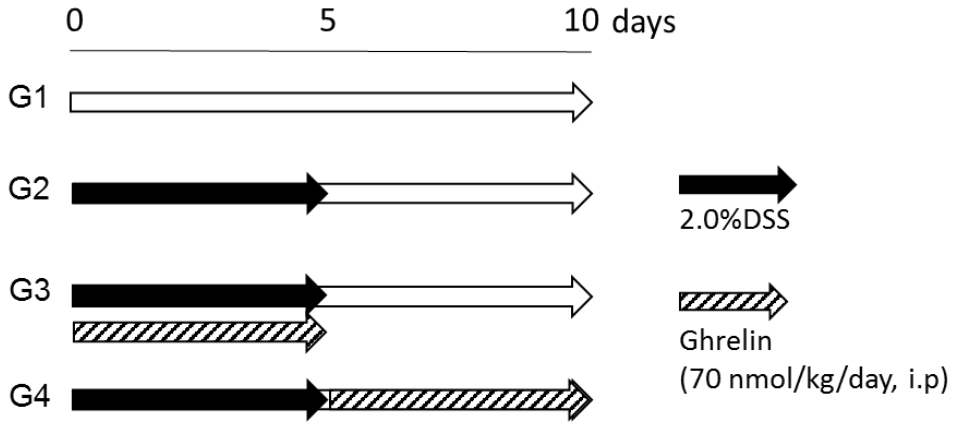

Fig. 1 The treatment protocol for the experiment (see Materials and Methods)

Fig. 2

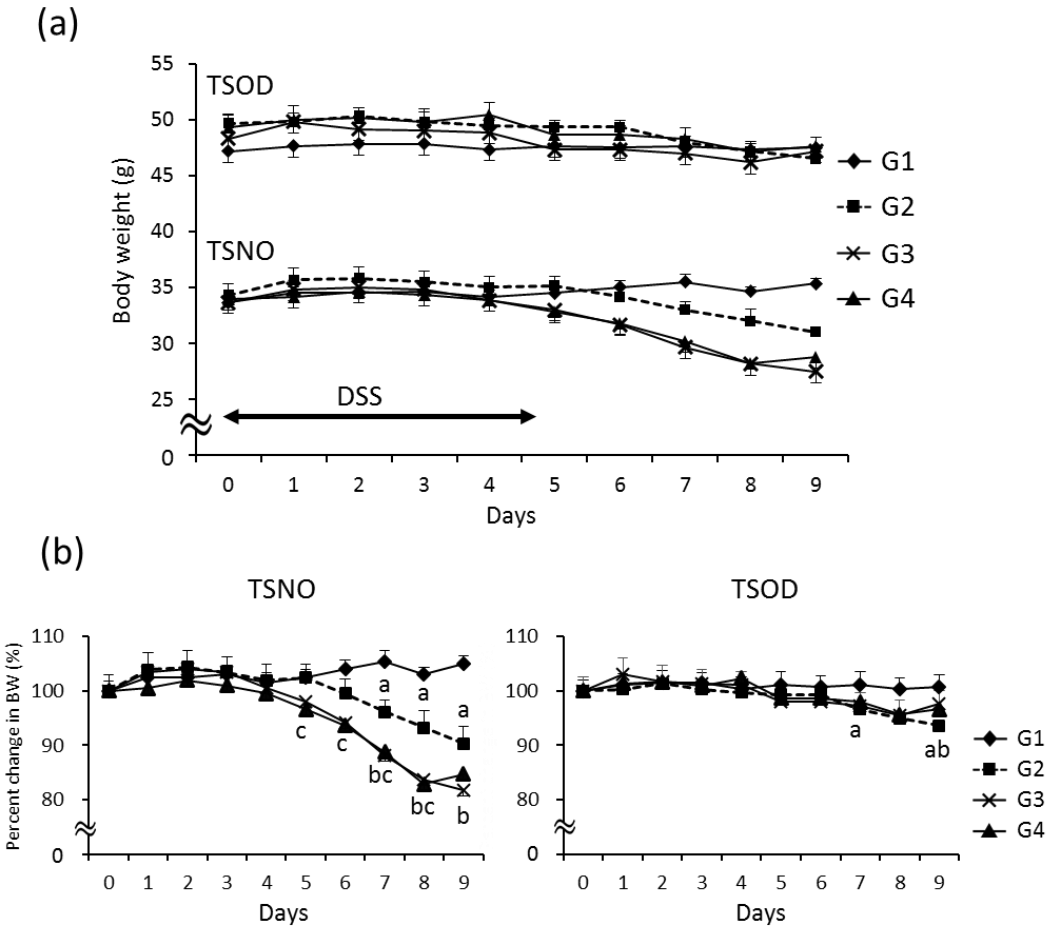

Fig. 2 Effect of ghrelin administration on body weight in TSNO (lean) and TSOD (obese) mice with DSS-induced colitis. Data from 6 mice are indicated for each group. (a) Upper 4 data indicate for TSOD mice of body weight. Lower 4 data indicate for TSNO mice of body weight. (b) Percent change in body weight. $^{a}P< 0.05$ between G1 and G2. $^{b}P< 0.05$ between G2 and G3. $^{c}P< 0.05$ between G2 and G4.

Fig. 3

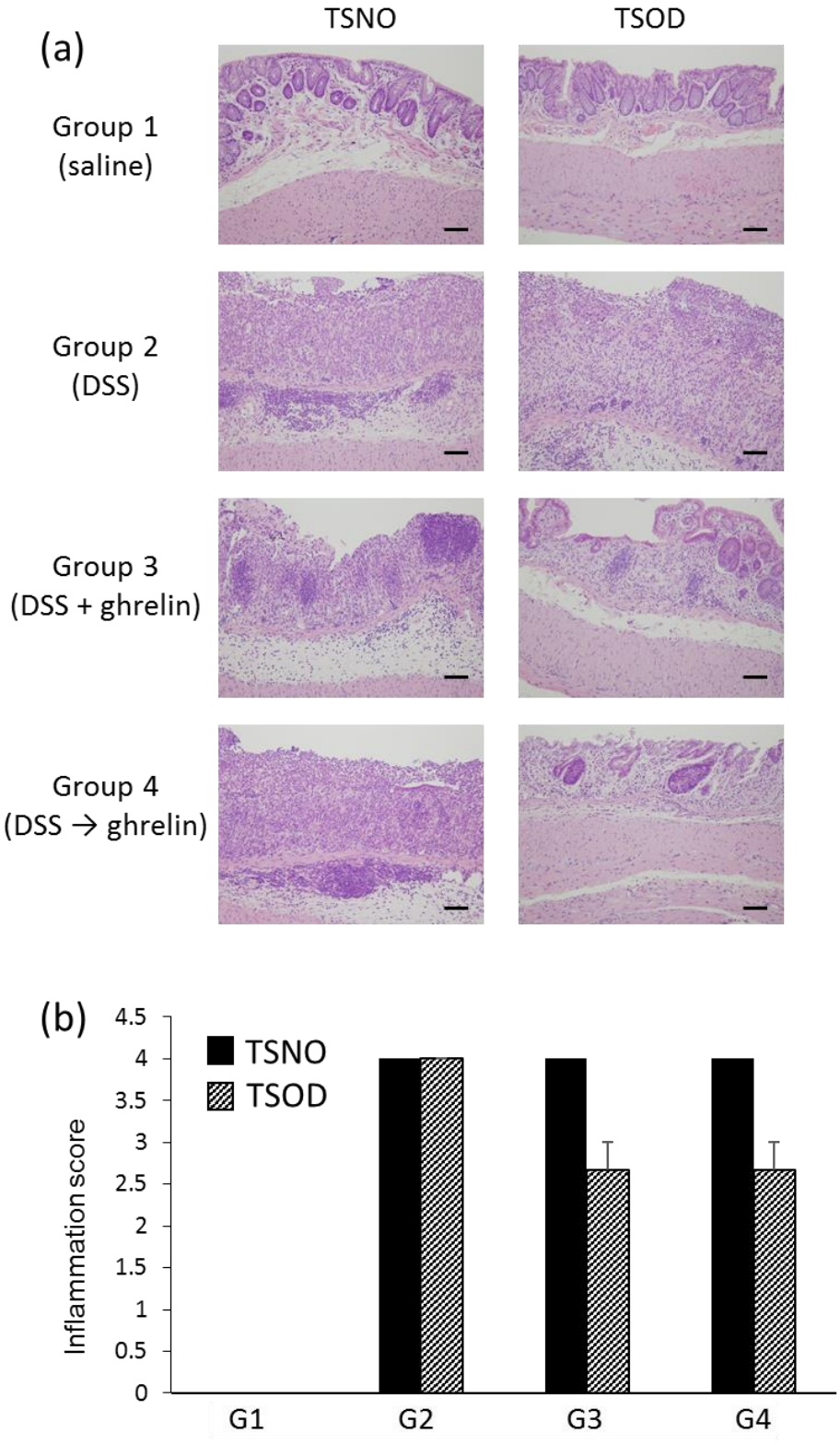

Fig. 3 Histological analysis of distal colon in day 10 (a) Histology of the distal colon (H&E stain). Bar, 0.1 mm. (b) Inflammation score. Data from 3 mice are indicated for each group.

Fig. 4

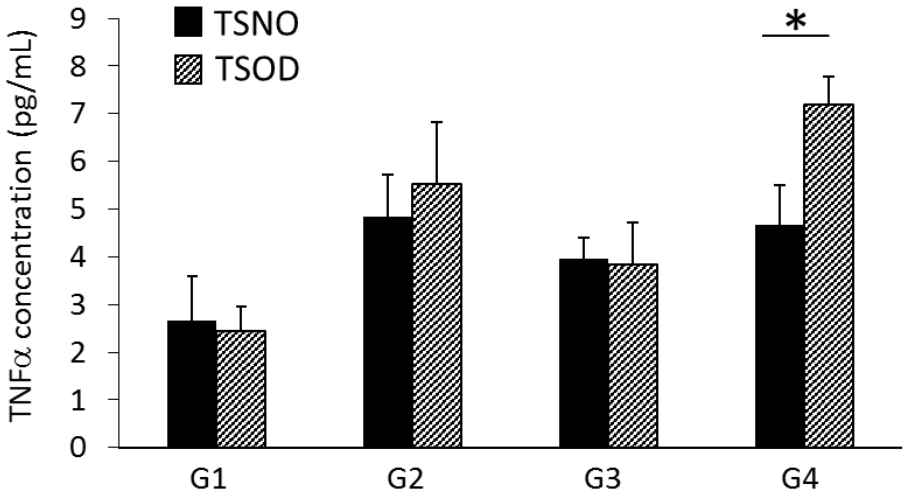

Fig. 4 Plasma TNFα concentration (mean±SEM) of each group in TSNO and TSOD mice. Data from 4 to 6 mice are indicated for each group. **P*< 0.05. Significant difference between TNSO and TSOD mice.

Fig. 5

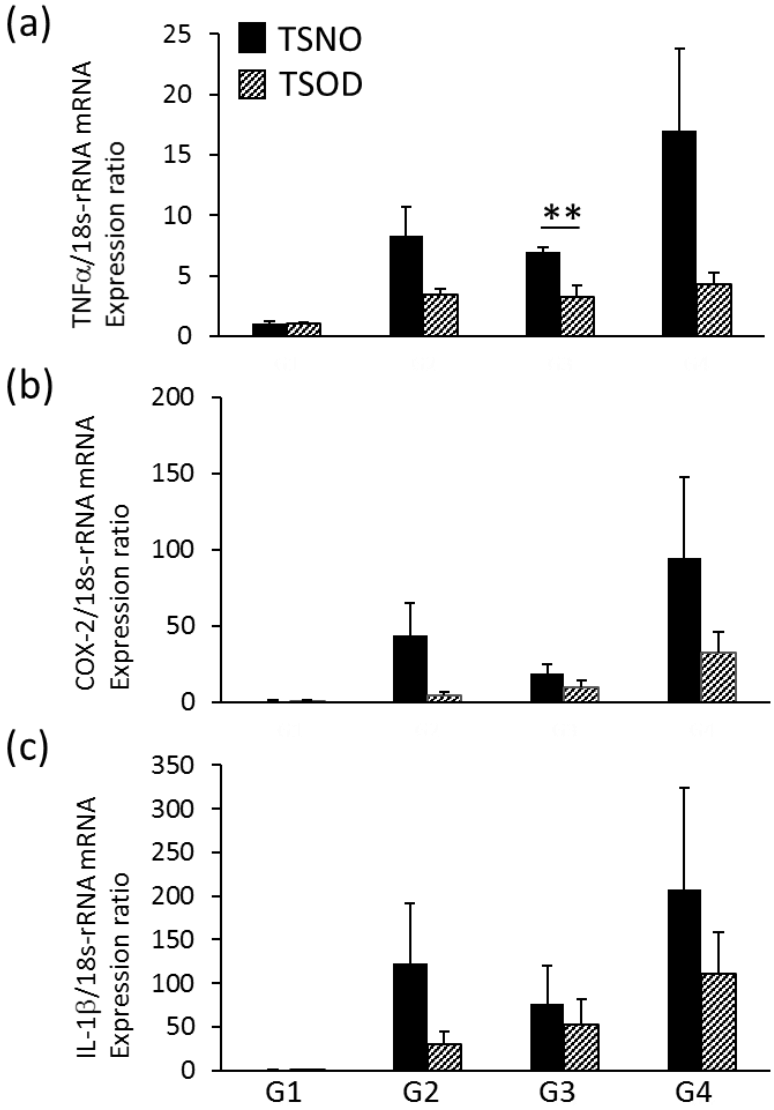

Fig. 5 Quantification of proinflammatory cytokine mRNA levels by real-time RT-PCR in colon tissue (mean±SEM, n=3 to 6 for each group). Whole mRNA levels are indicated for that of G1 converted in to one. (a): TNFα, (b): COX-2, (c): IL-1β. ***P*< 0.01. Significant difference between TNSO and TSOD mice.