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RESEARCH PAPER

Anti-inflammatory effects of phytosteryl ferulates in colitis induced by dextran sulphate sodium in mice

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Background and purpose: We have recently reported that phytosteryl ferulates isolated from rice bran inhibit nuclear factor-κB (NF-κB) activity in macrophages. In the present study, we investigated the effect of γ-oryzanol (γ-ORZ), a mixture of phytosteryl ferulates, cycloartenyl ferulate (CAF), one of the components of γ-ORZ, and ferulic acid (FA), a possible metabolite of γ-ORZ *in vivo*, on a model of colitis in mice.
Experimental approach: We induced colitis with dextran sulphate sodium (DSS) in mice and monitored disease activity index (DAI), histopathology score, tissue myeloperoxidase (MPO) activity, mRNA expressions of cytokines and COX-2, colon length, antioxidant potency and NF-κB activity in colitis tissue.
Key results: Both DAI and histopathology score revealed that DSS induced a severe mucosal colitis, with a marked increase in the thickness of the muscle layer, distortion and loss of crypts, depletion of goblet cells and infiltration of macrophages, granulocytes and lymphocytes. MPO activity, pro-inflammatory cytokines and COX-2 levels, NF-κB p65 nuclear translocation and inhibitory protein of nuclear factor-κB-α degradation levels were significantly increased in DSS-induced colitis tissues. γ-ORZ (50 mg kg^{-1} day^{-1} p.o.) markedly inhibited these inflammatory reactions and CAF had a similar potency. *In vitro* assay demonstrated that γ-ORZ and CAF had strong antioxidant effects comparable to those of α-tocopherol.
Conclusions and implications: Phytosteryl ferulates could be new potential therapeutic and/or preventive agents for gastrointestinal inflammatory diseases. Their anti-inflammatory effect could be mediated by inhibition of NF-κB activity, which was at least partly due to the antioxidant effect of the FA moiety in the structure of phytosteryl ferulates.
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Keywords: phytosteryl ferulates; γ-oryzanol; cycloartenyl ferulate; colitis; pro-inflammatory cytokines; NF-κB; antioxidant

Abbreviations: CAF, cycloartenyl ferulate; DAI, disease activity index; DSS, dextran sulphate sodium; FA, ferulic acid; IBD, inflammatory bowel disease; IL, interleukin; IκB, inhibitory protein of nuclear factor-κB; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; TNF-α, tumour necrosis factor-α; γ-ORZ, γ-oryzanol

Introduction

Ulcerative colitis and Crohn's disease are major forms of inflammatory bowel disease (IBD) (Melgar *et al*., 2005), which affect millions of people worldwide and are characterized by chronic uncontrolled inflammation of intestinal mucosa (Papadakis and Targan, 2000). These two forms of IBD comprise a widespread health hazard in modern society (Hanauer and Present, 2003).The pathogenesis of IBD remain poorly understood; however, the imbalance between pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interferon -γ, interleukin-1 (IL-1), IL-6, IL-12 and anti-inflammatory cytokines such as IL-4, IL-10, IL-11 is thought to play a pivotal role in modulating inflammation (Ardizzone and Bianchi Porro, 2005). These inflammatory responses begin with an infiltration of neutrophils and macrophages (Hanauer, 2006) and activated macrophages produce a potent mixture of broadly active inflammatory cytokines, including TNF-α, IL-1 and IL-6 (Podolsky, 2002). The conventional medical treatment of IBD relies on the use of aminosalicylates, corticosteroids, immunosuppressive drugs (azathioprine, 6-mercaptopurine, methotrexate, cyclosporin) and antibiotics (Baert *et al*., 2004). However, aminosalicylates (5-amino salicylic acid derivatives) and/or glucocorticoids remain the principal therapies for IBD at different stages of the disease (Podolsky, 2003; Domenech, 2006). 5-Amino salicylic acid-based agents are usually well tolerated but frequently induce side effects, such as acute pancreatitis, abdominal pain, diarrhoea, nausea, headache, anaemia, renal failure and anaphylaxis. Glucocorticoids are

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also commonly used to treat IBD patients; however, the clinical effects are often transitory, and disease recurs on reducing the dose, whereas high doses are accompanied by serious side effects, such as Cushing's syndrome. Moreover, many patients who respond to glucocorticoids become dependent on therapy and these patients cannot be withdrawn from treatment without a return of symptoms. Other agents used for IBD are immunomodulators such as thiopurines or infliximab; however, these agents still have serious side effects or clinical limitations for use. Therefore, there is a need for better therapeutic agents that effectively induce remission and alter the natural course of the disease with minimum or no side effects of the treatment.

Recently, considerable attention has been devoted on identifying naturally occurring chemopreventive phenolic substances, particularly those present in dietary and medicinal plants. Most of these naturally occurring phenolics retain antioxidative and anti-inflammatory properties, which contribute to their chemopreventive or chemoprotective activity (Surh, 1999). Rice bran is a component of raw rice that is obtained when it is removed from the starchy endosperm in the milling process. Rice bran oil derived from rice bran has been found to possess promising health-related benefits in the prevention of different diseases, including cancer, hyperlipidemia, fatty liver, hypercalciuria, kidney stones and heart disease (Jariwalla, 2001). Rice bran oil is a rich source of γ-oryzanol (γ-ORZ), which contains a number of phytosteryl ferulates, such as 24-methylenecycloartanyl ferulate, cycloartenyl ferulate (CAF), campesteryl ferulate, β-sitosteryl ferulate and campestanyl ferulate (Xu and Godber, 1999; Figure 1). It is particularly important that γ-ORZ exhibits antioxidant properties in *in vitro* systems (Hiramitsu and Armstrong, 1991; Kim *et al*., 1995; Xu *et al*., 2001). This free radical scavenging ability and antioxidant potency make γ-ORZ, a good candidate for pharmaceutical and cosmetic formulations and food (Juliano *et al*., 2005). The transcription factor, nuclear factor-κB (NF-κB), is involved in the regulation of pro-inflammatory genes and activation of this key factor regulates the expression of crucial mediators involved in chronic inflammatory disease and cancer, including proinflammatory cytokines, chemokines, adhesion molecules, COX-2 and inducible NOS (Tak and Firestein, 2001; Li and Verma, 2002). Consequently, agents that inhibit the activation of this transcription factor have the potential for therapeutic interventions. It is important to note that we have recently found some components of γ-ORZ, such as CAF, inhibiting NF-κB activity in macrophages (Nagasaka *et al*., 2007). Therefore, it seemed reasonable to assume that γ-ORZ could also be used for pharmaceutical purposes.

During the last decade, several experimental animal models of IBD have been developed to define different components of the pathophysiological processes that characterize these disorders (Elson *et al*., 1996; Panwala *et al*., 1998; Mayer and Collins, 2002; Jurjus *et al*., 2004; Ohda *et al*., 2005). Among these models, oral administration of dextran sulphate sodium (DSS) has been widely used to study the mechanisms of colonic inflammation and to evaluate the effect of any candidate drug for IBD (Aharoni *et al*., 2006).

We therefore decided to investigate the effect of γ-ORZ, a mixture of phytosteryl ferulates isolated from rice bran, on DSS-induced colonic inflammation in mice. We also investigated the effect of CAF, a major component of γ-ORZ, and ferulic acid (FA), a possible metabolite of phytosteryl ferulates *in vivo*. We found that phytosteryl ferulates might be good candidates to be used for the treatment of IBD.

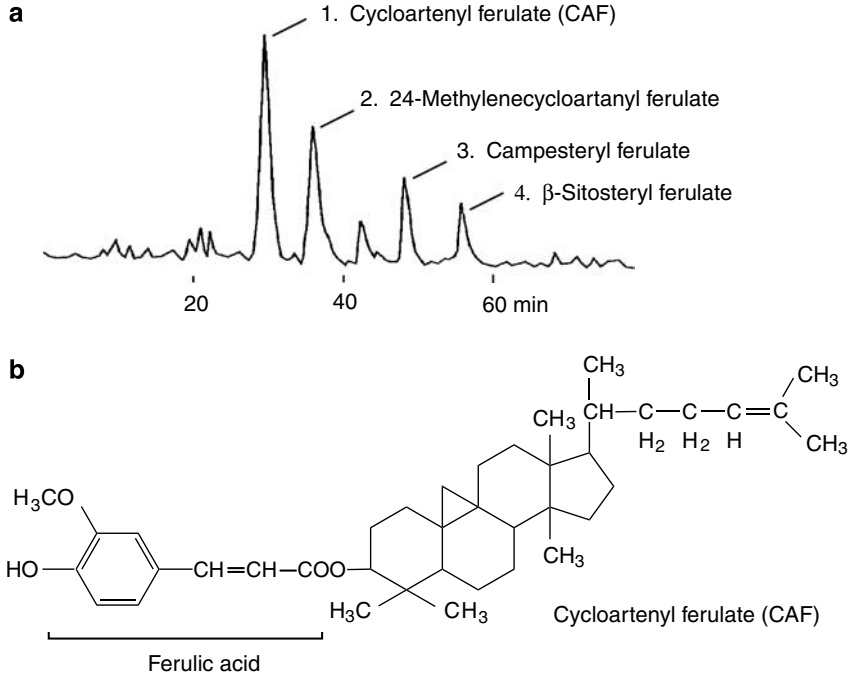

Figure 1 γ-ORZ, a mixture of phytosteryl ferulates isolated from rice bran (**a**), and its major component, CAF (**b**). HPLC elution pattern indicates that γ-ORZ contains at least four phytosteryl ferulates; that is (1) CAF; (2) 24-methylenecycloartanyl ferulate; (3) campesteryl ferulate and (4) β-sitosteryl ferulate. CAF, cycloartenyl ferulate; γ-ORZ, γ-oryzanol.

Materials and methods

Animals

Animal care and treatment were conducted in accordance with the institutional guidelines of the University of Tokyo. Experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Tokyo. Eight-week-old male C57BL/6J mice (Charles River, Yokohama, Japan) with an average weight of 24 g were used in this study. They were maintained under a constant 12-h light–dark cycle at an environmental temperature of 20–25 °C.

Induction of colitis

Experimental colitis was induced by giving mice drinking water *ad libitum* containing 1% (w/v) DSS for 6–16 days. Mice of each of the groups were monitored carefully every day to confirm that they consumed an approximately equal volume of DSS-containing water. For each experiment, mice were divided into three equal groups ($n = 6$–8). One group was kept as negative control and received drinking water without DSS throughout the experimental period. The mice receiving 1% DSS were given γ-ORZ, CAF or FA (50 mg kg^{-1} day^{-1} p.o.) daily for 8–18 days according to experimental design. Another group of mice receiving 1% DSS was given γ-ORZ (100 mg kg^{-1} day^{-1} s.c.) daily for 18 days according to experimental design and two other groups were offered vehicle only. All compounds were dissolved in vehicle (MilliQ water containing 0.01% Tween-20 and 0.5% carboxymethyl cellulose sodium) with sonication in an ice bath. Control groups were given vehicle daily for 8–18 days as appropriate. Administration of each drug was started 2 days before the DSS treatment. To examine therapeutic effect of γ-ORZ, colitis was induced by 3% (w/v) DSS for 5 days. After induction of colitis, γ-ORZ (50 mg kg^{-1} day^{-1}) or vehicle was administered orally, commencing on the sixth day and continuing up to day 16, according to experimental design.

Evaluation of disease activity index

Body weight, stool consistency and stool blood were recorded daily. Disease activity index (DAI) was determined by combining scores of (i) body weight loss, (ii) stool consistency and (iii) stool blood, divided by 3. Each score was determined as follows: change in body weight (0: <1%; 1: 1–5%; 2:5–10%; 3:10–15%, 4:>15%), stool blood (0: negative, 1: +, 2: ++, 3: +++, 4: ++++) and stool consistency (0: normal, 2: soft, 4: diarrhoea). Body weight loss was calculated as the percent difference between the original body weight (day 0) and the body weight on any particular day. Blood in faeces was detected with the occult blood test kit (Shionogi & Co. Ltd, Osaka, Japan). At the end of the experiment, mice were killed and the colon was separated from the proximal rectum, close to its passage under the pelvisternum. The colon length was measured between the ileo–cecal junction and the proximal rectum.

Histopathology

For histopathological analysis, a representative sample from the mid-part of the colon was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned (4 μm), stained with haematoxylin and eosin, and examined at × 200 magnification. The most affected part was scored, without the knowledge of the treatments. Inflammation was graded from 0 to 4 as follows in a blinded manner; 0: no signs of inflammation; 1: low leukocyte infiltration; 2: moderate leukocyte infiltration; 3: high leukocyte infiltration, thickening of the colon wall, moderate goblet cell loss, focal loss of crypts and 4: transmural infiltrations, massive loss of goblet cells, diffuse loss of crypts (Gonzalez-Rey *et al*., 2006).

For immunohistochemistry, 4 μm sections from the mid-colon were pretreated by boiling in citrate buffer (pH 6.1) in a microwave oven for 10 min and then cooling them at room temperature for 30 min. After cooling, sections were treated with 3% H_2O_2 for 5 min in dark. Nonspecific binding was blocked with 0.5% skim milk followed by incubation with rabbit polyclonal IgG against NF-κB (p65) and inhibitory protein of nuclear factor-κB (IκB)-α (Santa-Cruz Biotechnology, Tokyo, Japan) diluted 1:50 in Tris-buffered saline (TBS) containing 0.5% skim milk, overnight at 4 °C in a humidified chamber. After incubation with the primary antibody, the sections were washed three times with TBS and incubated with biotin-conjugated goat anti-rabbit IgG (diluted 1:100 in TBS containing 0.5% skim milk; Vector, Burlingame, CA, USA) for 2 h at room temperature, followed by avidin–biotin–peroxidase complexes (Vector). Peroxidase activity was visualized with the use of diaminobenzidine.

Myeloperoxidase assay

Myeloperoxidase (MPO) is an enzyme found in neutrophils and, in much smaller quantities, in monocytes and macrophages. The use of MPO activity as a marker of inflammatory cell infiltration was used as a convenient and valuable tool in evaluating the anti-inflammatory activity of γ-ORZ. Whole colon tissue was assessed for content and infiltration of polymorphonuclear cells. Tissues were homogenized in potassium phosphate buffer (pH 6.0) containing 0.3% hexadecyltrimenthyl ammonium bromide followed by three cycles of sonication and freeze thawing. Tissue particulate was discarded by centrifugation (20 000 *g*, 20 min) and supernatant was collected. Potassium phosphate buffer (pH 6.0; 618 μL) containing 0.5 mM *o*-dianisidine dihydrochloride (MP Biochemicals Inc., Osaka, Japan) was added to 125 μL of supernatant and 0.05% hydrogen peroxide. Changes in optical density were measured at 460 nm at room temperature (25 °C). MPO from human leukocytes was used as standard for this assay. MPO activity was expressed as μU per mg protein.

Determination of antioxidant activity with thiobarbituric acid reactive species assay

A modified thiobarbituric acid-reactive species assay was used to measure the potential antioxidant capacity using egg yolk homogenates as lipid rich media (Lin *et al*., 2002). All

the values were calculated on the basis of the percentage antioxidant index (AI %):

$$AI\,\% = (1 - A_T/A_C) \times 100$$

where A_C is the absorbance value of the fully oxidized control and A_T is the absorbance of the test sample.

RNA extraction and quantification of TNF-α, IL-1β, IL-6 and COX-2 mRNA

Total RNA from colon was extracted using Trizol reagent (Invitrogen, Tokyo, Japan). The concentration of RNA was adjusted to $1\,\mu g\,\mu L^{-1}$ with RNase-free distilled water. First-strand cDNA was synthesized using a random nine-mer primer and avian myeloblastosis virus Reverse Transcriptase XL at 30 °C for 10 min, 55 °C for 45 min, 99 °C for 5 min and 4 °C for 5 min. PCR amplification was performed using AmpliTaq Gold polymerase. The oligonucleotide primers for TNF-α designed from mouse (NM_013693) were ACGGC ATGGATCTCAAAGAC (forward) and CGGACTCCGCAAA GTCTA AG (reverse), for IL-1β (NM_008361) were TGACG TTCCCATTAGACAGC (forward) and TGGGGAAGGCATTA GAAACA (reverse), for IL-6 (NM_031168) were TCTCTGGGA AATCGTGGAAA (forward) and GATGGTCTTGGTCCTTAG CC (reverse) and for COX-2 (NM_011198.2) were AAGCCGA GCACCTTTGGAG (forward) and ATTGATGGTGGCTGTTTT GGTAG (reverse) and the suitable size of synthesized cDNA were 324, 497, 397 and 147 bp, respectively. The oligonucleotide primers for mouse glyceraldehyde-3-phosphate dehydrogenase used as a house-keeping gene designed from mouse were TGTTCCTACCCCCAATGTGT (forward) and CCCTGTTGCTGTAGCCGTAT (reverse), and the suitable size of the synthesized cDNA was 269 bp. After initial denaturation at 95 °C for 10 min, amplification at 95 °C for 45 s, 55 °C for 1.0 min and 72 °C for 1.45 min was performed using a thermal cycler (Takara PCR Thermal Cycler MP; Takara Biomedicals, Shiga, Japan). Initially, we checked PCR for 28 cycles, 30 cycles and 32 cycles and found similar results. Finally, we selected 32 cycles for reverse transcription-PCR analysis instead of amplification nature of RNA expression. PCR products were separated by electrophoresis on 2% agarose gel containing 0.1% ethidium bromide. Detectable fluorescent bands were visualized by an ultraviolet transilluminator using FAS III (TOYOBO, Tokyo, Japan). The results were expressed as the ratio of optical density to glyceraldehyde-3-phosphate dehydrogenase.

Extraction of cytoplasmic and nuclear proteins and western blot analysis

Nuclear proteins were extracted by the method of Martin *et al*. (2006). Briefly, frozen colonic tissues were weighed and homogenized in ice-cold hypotonic buffer (1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulphonyl fluoride, 1.0 mM dithiothreitol and 10 mM HEPES, pH 7.9). Homogenates were incubated for 10 min on ice and centrifuged (20 000 *g*, 20 min, 4 °C). Cytoplasmic proteins were collected from the supernatants and nuclear proteins from the pellets. Pellets were washed once with the same volume of hypotonic buffer as used for homogenization and centrifuged at 10 000 *g*, 15 min, 4 °C, after which they were suspended in ice-cold low-salt buffer. Nuclear proteins were released by adding a high-salt buffer drop by drop to a final concentration of 0.4 M KCl. Samples were incubated on ice for 30 min, with smooth shaking. Soluble nuclear proteins were recovered by centrifugation (20 000 *g*, 40 min, 4 °C) and proteins were stored at −80 °C. Protein concentration of the homogenate was determined with Bio-rad colorimetric method. Aliquots of supernatants containing equal amounts of protein (nuclear and cytoplasm) were separated on 10% acrylamide gel by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. In the next step, the proteins were electrophoretically transferred onto an Immun-Blot polyvinylidine difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA) and later incubated with primary antibody NF-κB p65 and IκB-α at a dilution of 1:200, respectively, in TBS containing 5% skim milk (Santa Cruz Biotechnology, Japan) overnight at 4 °C. The membrane was washed three times for 15 min and incubated with the secondary biotin-conjugated goat anti-rabbit IgG (diluted 1:1000 in TBS containing 5% skim milk; Vector) for 60 min at room temperature. After secondary antibody, the membranes were treated with streptavidin–horseradish peroxidase conjugate (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) for 30 min at room temperature. With a view to ensure equal loading, the blots were analysed for β-actin expression using an anti-β-actin monoclonal antibody (Sigma Aldrich, Tokyo, Japan). Immunodetection was performed using an enhanced chemiluminescence light-detecting kit (Amersham ECL plus Western Blotting Detection kit, GE Healthcare, Buckinghamshire, UK). Densitometric data were studied following normalization to the control (house-keeping gene).

Statistical analyses

Numerical data are expressed as mean ± s.e.mean. Statistical evaluation was carried out by Student's *t*-test and, where appropriate, one-way ANOVA; Tukey's all compare test was applied.

Drugs and reagents

Drugs and reagents used in the experiment were DSS, *o*-dianisidine dihydrochloride, cetyl trimenthyl ammonium bromide (MP Biomedicals Inc., Osaka, Japan), MPO from human leukocytes, 2-thiobarbituric acid (Sigma-Aldrich, Tokyo, Japan), occult blood slide shionogi II (Shionogi & Co. Ltd), 2-2′-azobis(2-methylpropionamidine) dihydrochloride, CAF and FA (Wako Chemical, Osaka, Japan). γ-ORZ was purified from Japanese rice, Koshihikari, as reported earlier (Bligh and Dyer, 1959).

Results

Evaluation of anti-inflammatory effects of γ-ORZ in DSS-induced colitis in mice

A combinatorial DAI, using body weight loss, stool consistency and stool blood, was used to analyse the therapeutic

benefit of γ-ORZ treatment. DSS (1%) administration was associated with significant clinical changes, including weight loss, appearance of occult faecal blood and diarrhoea in untreated mice. Treatment with γ-ORZ, 50 mg kg^{-1} day^{-1}, p.o., or 100 mg kg^{-1} day^{-1}, s.c., during the entire time course of DSS-treatment, suppressed the pathological conditions, including body weight loss, intestinal bleeding and diarrhoea, resulting in significant amelioration of intestinal inflammation ($P<0.01$–0.05) (Figures 2d and 3d).

γ-ORZ decreased histopathology scores in DSS-induced colitis in mice

Histopathological examination of the colon of mice given DSS in drinking water showed mucosal inflammation involving all layers of the bowel wall, with a marked increase in the thickness of muscle layer, crypts distortion and, in some sections, loss of crypts were evident. Extensive granulation tissues with the presence of monocytes and lymphocytes, depletion of goblet cells were apparent in the mucosa in untreated mice (Figures 2b and 3b). However, γ-ORZ (50 mg kg^{-1} day^{-1} p.o. or 100 mg kg^{-1} day^{-1} s.c. for 16 days) protected against both the infiltration of inflammatory cells and the mucosal damage, resulting in a significant reduction of histopathology scores (Figures 2c, e, f and 3c, e, f).

γ-ORZ attenuated MPO level in the DSS-induced colitis in mice

The histopathological study indicated that one mechanism underlying the protective effects of γ-ORZ involved a reduced infiltration of inflammatory cells into the colonic

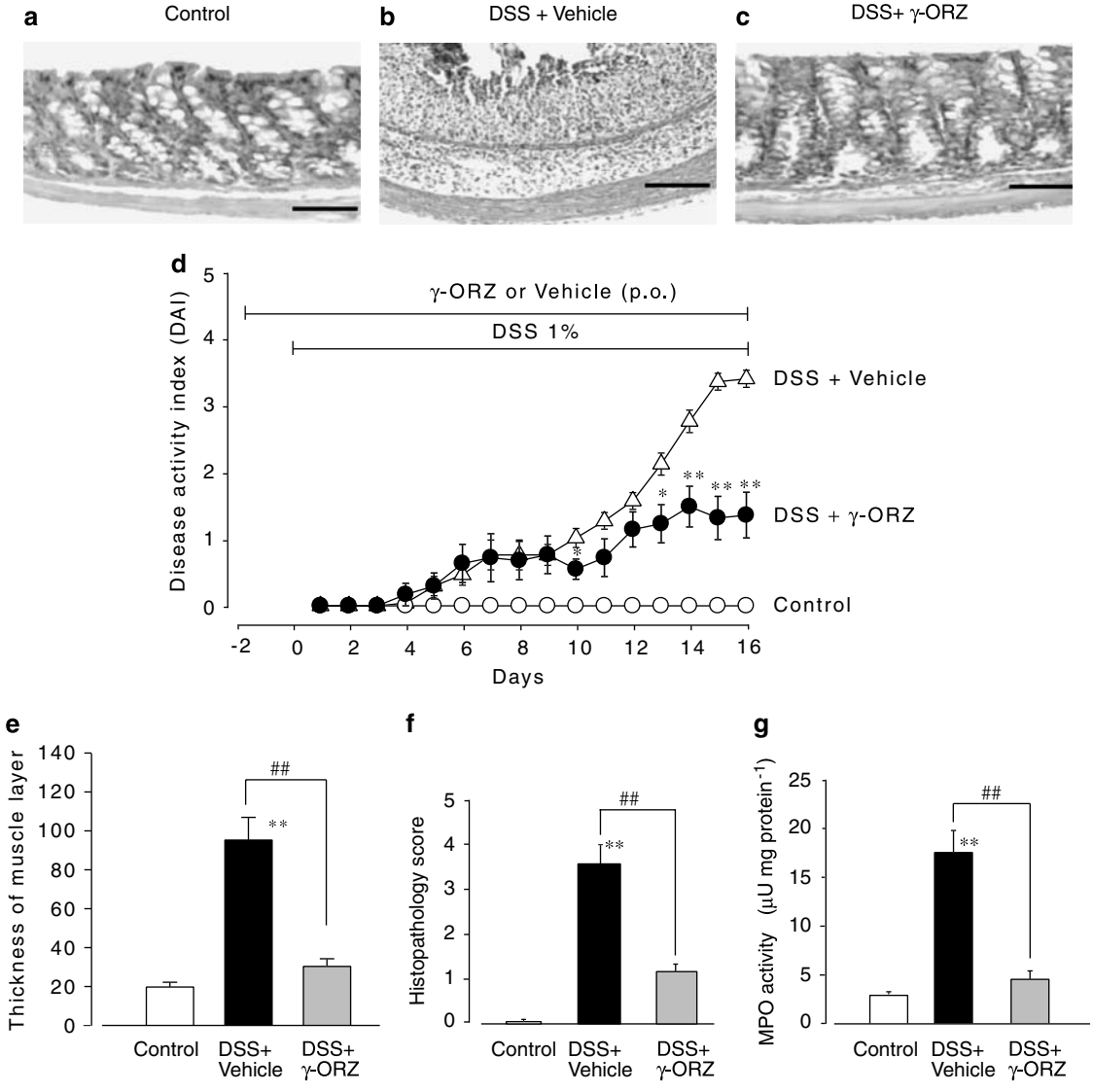

Figure 2 Oral administration (p.o.) of γ-ORZ ameliorates DSS-induced colitis in mice. Experimental colitis was induced by 1% DSS in drinking water (*ad libitum*) for 16 days with or without γ-ORZ (50 mg kg^{-1} day^{-1} p.o. up to 16 days). Histology (haematoxylin and eosin staining) of full thickness of mid-colon are shown in (**a**) non-treatment:control, (**b**) 1% DSS + vehicle and (**c**) 1% DSS + γ-ORZ. (**d**) DAI (*$P<0.05$, **$P<0.01$, significantly different from DSS treatment), (**e**) thickness of muscle layer, (**f**) histopathology score and (**g**) colonic MPO activity (details described in Materials and methods). **Significantly different from control ($P<0.01$). ##Significantly different between DSS + vehicle treatment and DSS + γ-ORZ treatment ($P<0.01$). Values are expressed as mean ± s.e.mean. $n=6$–8. Bar = 50 μm. DAI, disease activity index; DSS, dextran sulphate sodium; MPO, myeloperoxidase; γ-ORZ, γ-oryzanol.

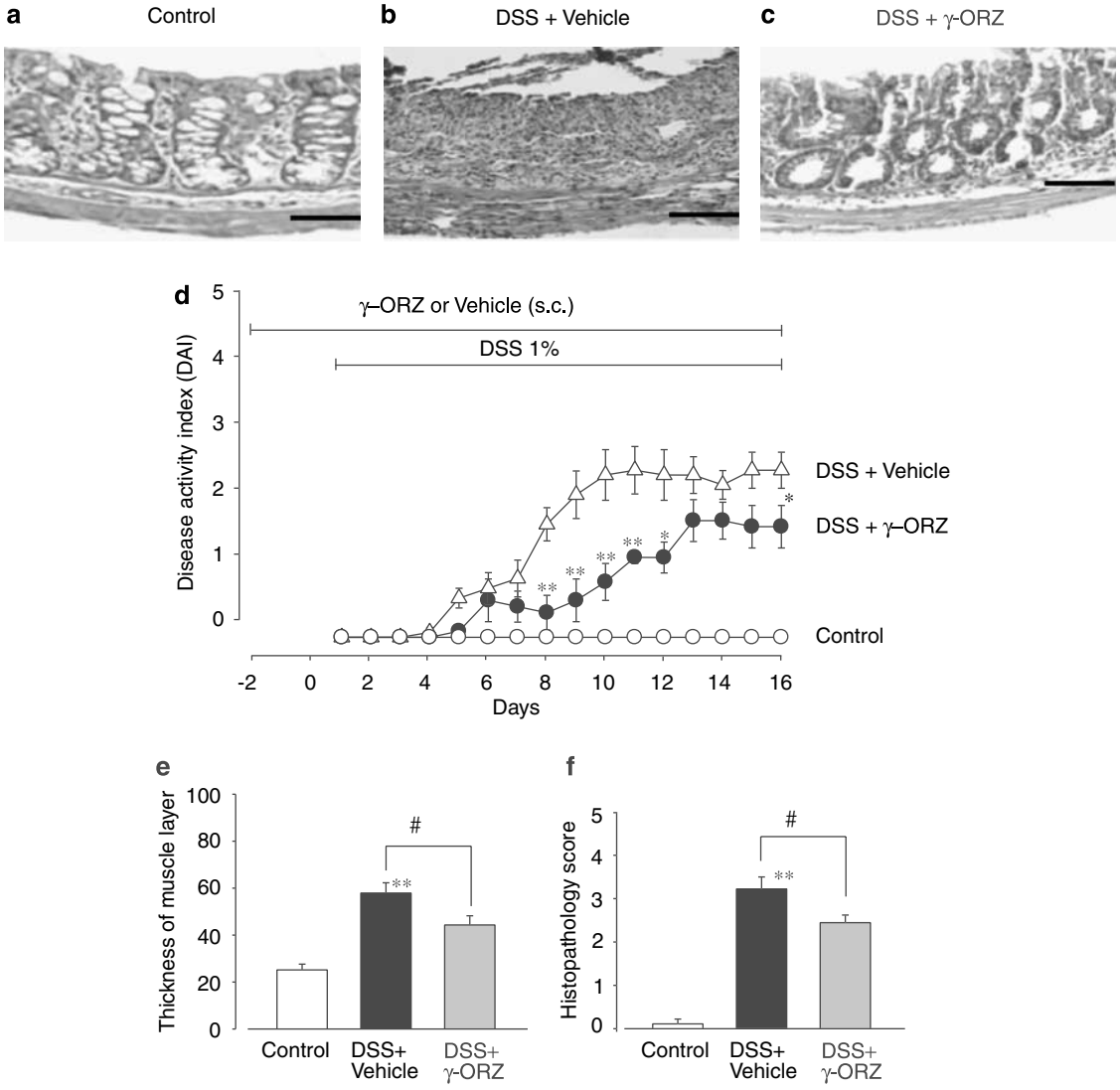

Figure 3 Subcutaneous administration of γ-ORZ ameliorates DSS-induced colitis in mice. Experimental colitis was induced by 1% DSS in drinking water (*ad libitum*) for 16 days with or without γ-ORZ (100 mg kg^{-1} day^{-1} s.c. up to 16 days). Histology (haematoxylin and eosin staining) of full thickness of mid-colon are shown in (**a**) Non-treatment:control, (**b**) 1% DSS + vehicle and (**c**) 1% DSS + γ-ORZ. (**d**) DAI (*$P<0.05$, **$P<0.01$, significantly different from DSS treatment), (**e**) thickness of muscle layer, (**f**) histopathology score. **Significantly different from control ($P<0.01$). #Significantly different between DSS + vehicle treatment and DSS + γ-ORZ treatment ($P<0.05$). Values are expressed as mean ± s.e.mean. $n=5$–6. Bar = 50 μm. DAI, disease activity index; DSS, dextran sulphate sodium; γ-ORZ, γ-oryzanol.

mucosa. Thus, colon inflammation was quantitatively assayed by assessment of MPO activity. We found that MPO activity was correlated with the development of colonic inflammation and that administration of γ-ORZ (50 mg kg^{-1} day^{-1} p.o. for 16 days) significantly suppressed MPO activity in colonic tissue (Figure 2g).

γ-ORZ suppressed expression of mRNA for inflammatory cytokines and COX-2 in DSS-induced colitis

Using semiquantitative reverse transcription-PCR, the levels of mRNA of pro-inflammatory cytokines in mice with DSS-induced colitis treated with γ-ORZ was evaluated and compared with those in untreated mice with colitis and with vehicle-treated control mice. The changes in mRNA expression of TNF-α, IL-1β, IL-6 and COX-2 in colon (relative ratio to glyceraldehyde-3-phosphate dehydrogenase) are shown in Figure 4 and demonstrate that the expression of these inflammatory cytokines and COX-2 was significantly greater in DSS-treated mice. Notably, it was time dependent and the expression level was very high on day 8 and decreased on day 16, but still remained higher than those of control mice. γ-ORZ significantly reduced the upregulated expression of IL-1β, IL-6, TNF-α and COX-2 mRNA in mice with DSS-induced colitis.

γ-ORZ inhibited activation and nuclear translocation of NF-κB in DSS-induced colitis

As NF-κB activity plays a critical role in the inflammation, we examined the effect of γ-ORZ on this transcription factor in the inflamed tissue. Immunohistopathology showed that

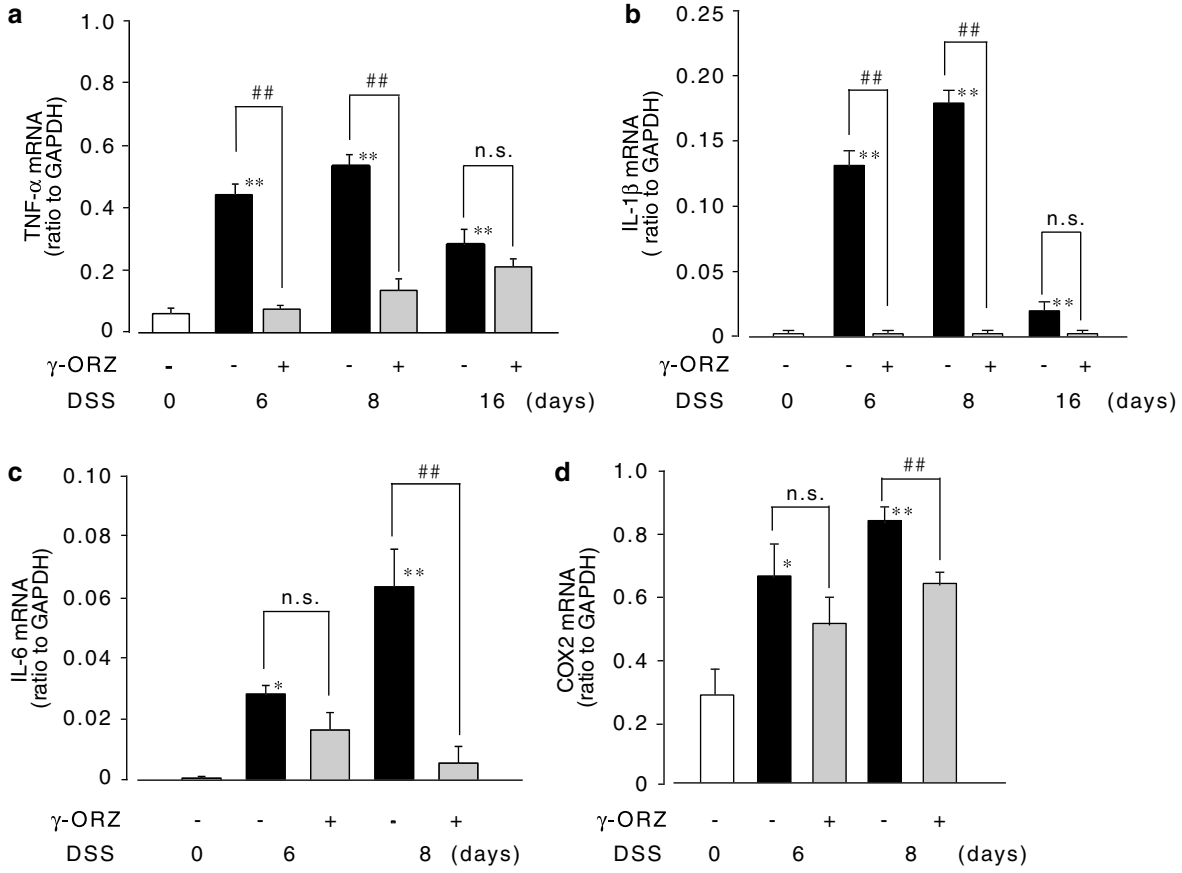

Figure 4 Changes in the mRNA expression of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, and COX-2 in mice with DSS-induced colitis. Mice were given 1% DSS in drinking water *ad libitum* for 6, 8 and 16 days with or without γ-ORZ (50 mg kg^{-1} day^{-1} p.o.). The expression of mRNA for TNF-α, IL-1β, IL-6 (day 6 and 8) and COX-2 (day 6 and 8) in colitis tissues are significantly greater than control (day 0) (*$P<0.05$ and **$P<0.01$). ##Significantly different between DSS + vehicle treatment and DSS + γ-ORZ treatment ($P<0.01$). NS, not significantly different from control or between DSS + vehicle treatment and DSS + γ-ORZ treatment. $n=4$. DSS, dextran sulphate sodium; IL, interleukin; TNF-α, tumour necrosis factor-α; γ-ORZ, γ-oryzanol.

NF-κB p65 subunit was strongly stained not only in cytoplasm but also in nuclei of epithelial cells as well as inflammatory cells in the DSS-treated mice compared with control mice (Figures 5A-a, -b), indicating the activation of NF-κB. On the other hand, IκB-α was more clearly observed in control mice compared with DSS-treated mice (Figures 6A-a, -b). The staining pattern of NF-κB p65 and IκB-α was returned to normal upon treatment with γ-ORZ (50 mg kg^{-1} day^{-1} p.o.) (Figures 5A–c and 6A–c).

We also analysed nuclear translocation of NF-κB p65 and the amount of cytoplasmic IκB-α using western blot analysis. As demonstrated in Figure 5B, the nuclear NF-κB p65 significantly increased in the colitis tissue and was significantly reduced by treatment with γ-ORZ (50 mg kg^{-1} day^{-1} p.o.). Consequently, the level of IκB-α in the cytoplasmic extract significantly decreased in colitis tissue, which was significantly increased by treatment with γ-ORZ (50 mg kg^{-1} day^{-1} p.o.) (Figure 6B).

Effects of CAF and FA on the DSS-induced colitis

γ-Oryzanol is a mixture of phytosteryl ferulates derived from rice bran oil, including CAF, 24-methylenecycloartanyl ferulate, campesteryl ferulate, β-sitosteryl ferulate and campestanyl ferulate as major components (Xu and Godber, 1999) (Figure 1). In addition, FA, an antioxidant, may be produced as a metabolite of γ-ORZ after *in vivo* administration. Therefore, in this study, we investigated the effect of CAF and FA on DSS-induced colitis. As shown in Figure 7, CAF (50 mg kg^{-1} day^{-1} p.o.) significantly suppressed DAI, histopathology score and MPO activity as well as γ-ORZ. FA (50 mg kg^{-1} day^{-1} p.o.) also suppressed these inflammatory parameters; however, the anti-inflammatory effects were weaker than those of γ-ORZ and CAF (note that FA only inhibited the DAI at day 16).

Antioxidant activity of γ-ORZ in lipid media

We evaluated the antioxidant activity of γ-ORZ, CAF and FA *in vitro*, using the thiobarbituric acid-reactive species method (Lin *et al*., 2002). The inhibitory effects on lipid peroxidation of γ-ORZ and CAF were comparable with those of the established antioxidant, α-tocopherol. However, FA also had antioxidant activity but was much less potent, approximately equal to that of ascorbic acid (Figure 8).

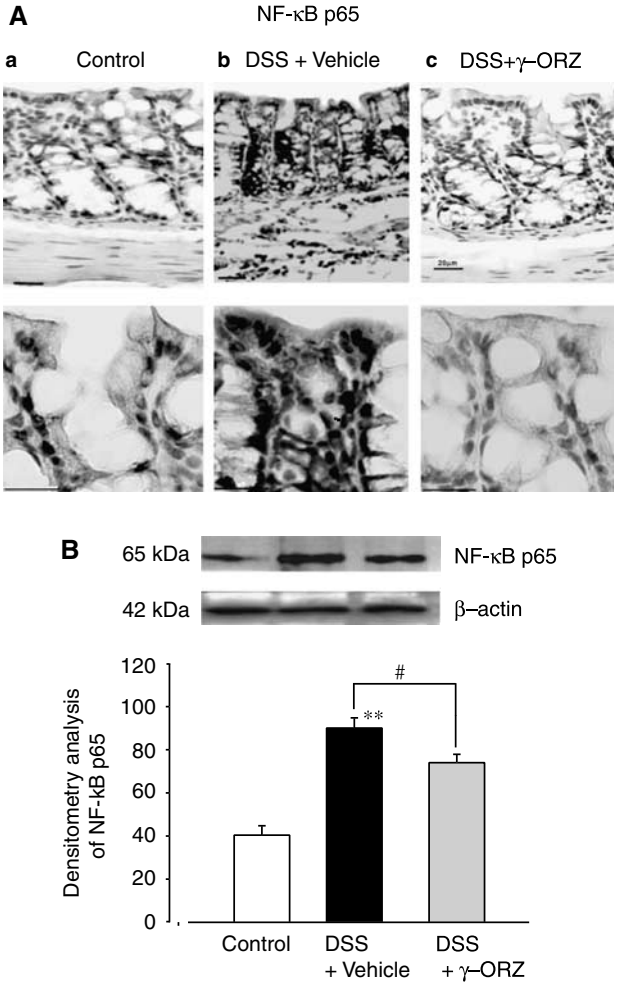

Figure 5 Effect of γ-ORZ on NF-κB activity in DSS-induced colitis. Mice were given 1% DSS in drinking water *ad libitum* for 8 days with or without γ-ORZ (50 mg kg^{-1} day^{-1} p.o.). (**A**) Immunohistopathology of NF-κB p65 of mid-colon (control, DSS + vehicle treatment and DSS + γ-ORZ treatment). Untreated colitis tissues exhibited NF-κB p65 translocation in the nuclei of epithelial cells as well as in the infiltrated inflammatory cells (**b**). γ-ORZ prevented the staining pattern of NF-κB p65 observed in colitis tissues (**c**). (**B**) Western blotting of NF-κB p65 of mid-colon. The upper panel shows an example of western blot following probing with the antibody to NF-κB p65. The lower histogram is the data derived from the western blots following densitometry analysis. **Significantly different from control with $P<0.01$. #Significantly different between DSS + vehicle treatment and DSS + γ-ORZ treatment ($P<0.05$). $n=4$–6. DSS, dextran sulphate sodium; NF-κB, nuclear factor-κB; γ-ORZ, γ-oryzanol.

Therapeutic effect γ-ORZ in DSS-induced colitis

Finally, we investigated the therapeutic effect of γ-ORZ on DSS-induced colitis. The therapeutic effect of γ-ORZ was investigated in the colitis model induced by 3% DSS given for 5 days. In this model, γ-ORZ (50 mg kg^{-1} day^{-1} p.o.) or vehicle treatment was started on the sixth day after DSS. A summary of the therapeutic effects is provided in Figure 9. γ-ORZ (50 mg kg^{-1} day^{-1} p.o.) significantly reduced DAI and histopathology score, MPO activity and inhibited shortening of the colon. Significant inhibition of DAI was observed from day 9 to 16.

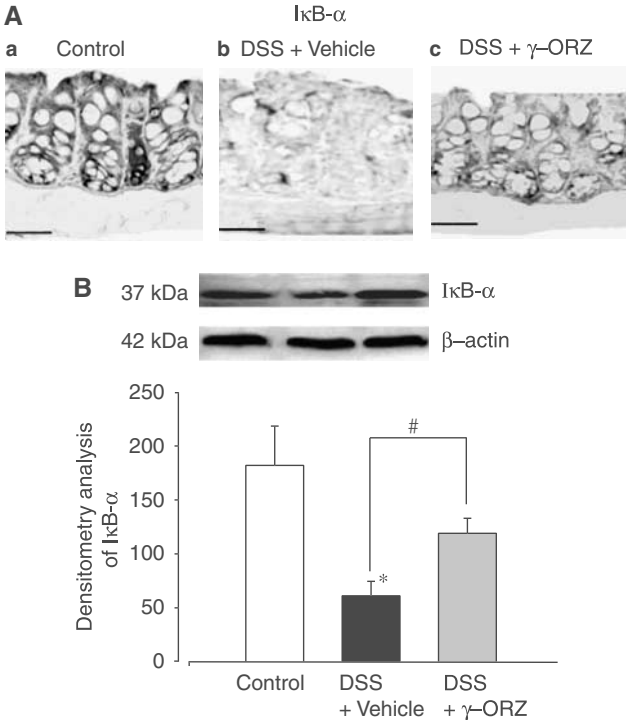

Figure 6 Effect of γ-ORZ on IκB-α expression in DSS-induced colitis. Mice were given 1% DSS in drinking water *ad libitum* for 8 days with or without γ-ORZ (50 mg kg^{-1} day^{-1} p.o.). (**A**) Immunohistopathology of IκB-α of mid-colon (control, DSS + vehicle treatment and DSS + γ-ORZ treatment). DSS-induced untreated colitis tissues exhibited remarkable degradation of IκB-α (**b**), and γ-ORZ prevented this decrease (**c**). (**B**) Western blotting of IκB-α of mid-colon. The upper panel shows an example of western blot following probing with the antibody for IκB-α. The lower histogram is the data derived from the western blots following densitometry analysis, with the results shown as IκB-α level compared with the untreated group. *Significantly different from control with $P<0.05$. #Significantly different between DSS + vehicle treatment and DSS + γ-ORZ treatment ($P<0.05$). $n=4$–6. DSS, dextran sulphate sodium; IκB, inhibitory protein of nuclear factor-κB; γ-ORZ, γ-oryzanol.

Discussion

To investigate the therapeutic effect of γ-ORZ on IBD, we selected a model of colitis induced by DSS in mice. This model exhibits symptoms comparable to those of human ulcerative colitis (Strober *et al*., 2002), such as diarrhoea, bloody faeces, body weight loss, mucosal ulceration and shortening of colon length (Kwon *et al*., 2008). We administered γ-ORZ orally or subcutaneously, during DSS consumption (co-administration) or after DSS consumption (post-administration) in mice. In this experiment, we measured the severity of clinical colitis by assessing the body weight loss, stool consistency and stool blood, and finally, evaluated the therapeutic effects of γ-ORZ treatment. Our findings demonstrate that γ-ORZ treatment significantly suppressed DSS-induced colitis in mice, improving their body weight and stool consistency as well as decreasing intestinal bleeding. In addition, the DSS-induced colitis exhibited mucosal inflammation with extensive depletion of goblet cells in the mucosa. γ-ORZ treatment greatly reduced the infiltration of leukocytes and mucosal

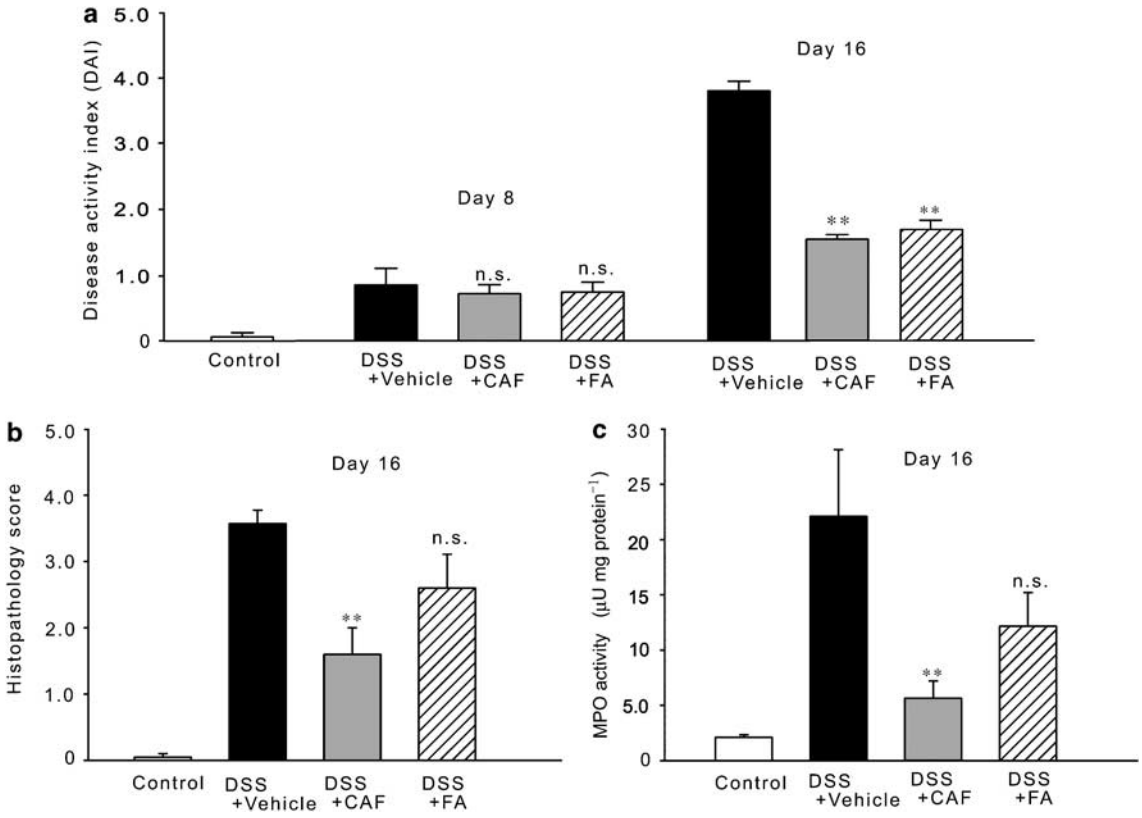

Figure 7 Effect of CAF and FA (50 mg kg^{-1} day^{-1} p.o. for 8–16 days) on DAI, histopathologic score (HS) and MPO activity in mice with DSS-induced colitis (details in the Materials and methods). **Significantly different from DSS treatment group ($P<0.01$). NS, not significantly different from DSS treatment group. $n=6$–8. CAF, cycloartenyl ferulate; DAI, disease activity index; DSS, dextran sulphate sodium; FA, ferulic acid; MPO, myeloperoxidase.

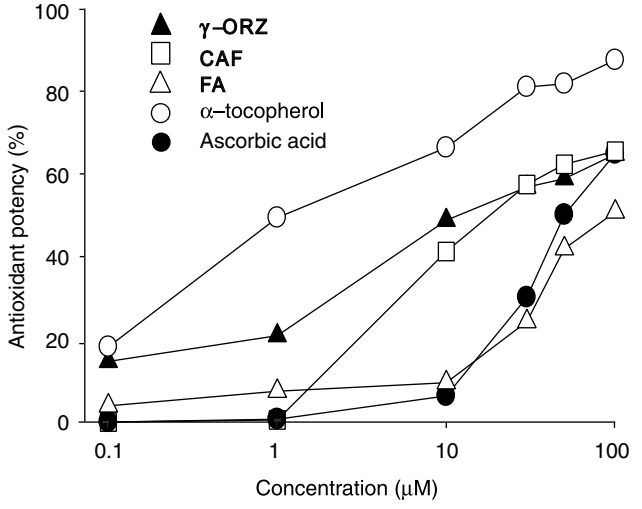

Figure 8 Antioxidant activity of γ-ORZ, FA and CAF (0.1–100 μM) in comparison with α-tocopherol and ascorbic acid. Oxidation was measured using the thiobarbituric acid-reactive species assay (see Materials and methods). The data are the means from three individual experiments. Estimated molecular weight was employed for γ-ORZ (MW: 603). CAF, cycloartenyl ferulate; FA, ferulic acid; γ-ORZ, γ-oryzanol.

damage, resulting in significant amelioration of histopathology scores and preserving colon length (Figures 2a–g, 3a–f and 9a–h).

Pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α are induced by the immunocompetent cells after activation of NF-κB in colitis tissue (Monteleone *et al*., 2002). The roles of these pro-inflammatory cytokines, particularly TNF-α, in the pathogenesis of IBD are well documented both in naturally occurring disease (Murch *et al*., 1993; Breese *et al*., 1994; van Dullemen *et al*., 1995; Plevy *et al*., 1997; Baert *et al*., 1999; D'Haens *et al*., 1999) and in animal models of colitis (Rojas-Cartagena *et al*., 2005;Kinoshita *et al*., 2006). These pro-inflammatory cytokines amplify the inflammatory cascade of inflammatory mediators, destructive enzymes and free radicals that cause tissue damage (Podolsky, 2002; Kurtovic and Segal, 2004). In the present study, we observed an elevation of the proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, in DSS colitis tissue. However, oral administration of γ-ORZ greatly inhibited the transcripts of these cytokines (Figures 4a–c). Eicosanoids also play a central role in the intestinal inflammation. IL-1β, a key cytokine associated with intestinal mucosal inflammation, induced COX-2 expression in experimental colitis, and the increased prostaglandin E_2 secretion is associated with IBD (Sheibanie *et al*., 2007). The present results demonstrated that γ-ORZ significantly reduced COX-2 expression in DSS-induced colitis (Figure 4d).

γ-Oryzanol derived from rice bran oil is a mixture of phytosteryl ferulates, which comprises at least four components: 24-methylenecycloartanyl ferulate, CAF, β-sitosteryl ferulate and campestanyl ferulate. In this

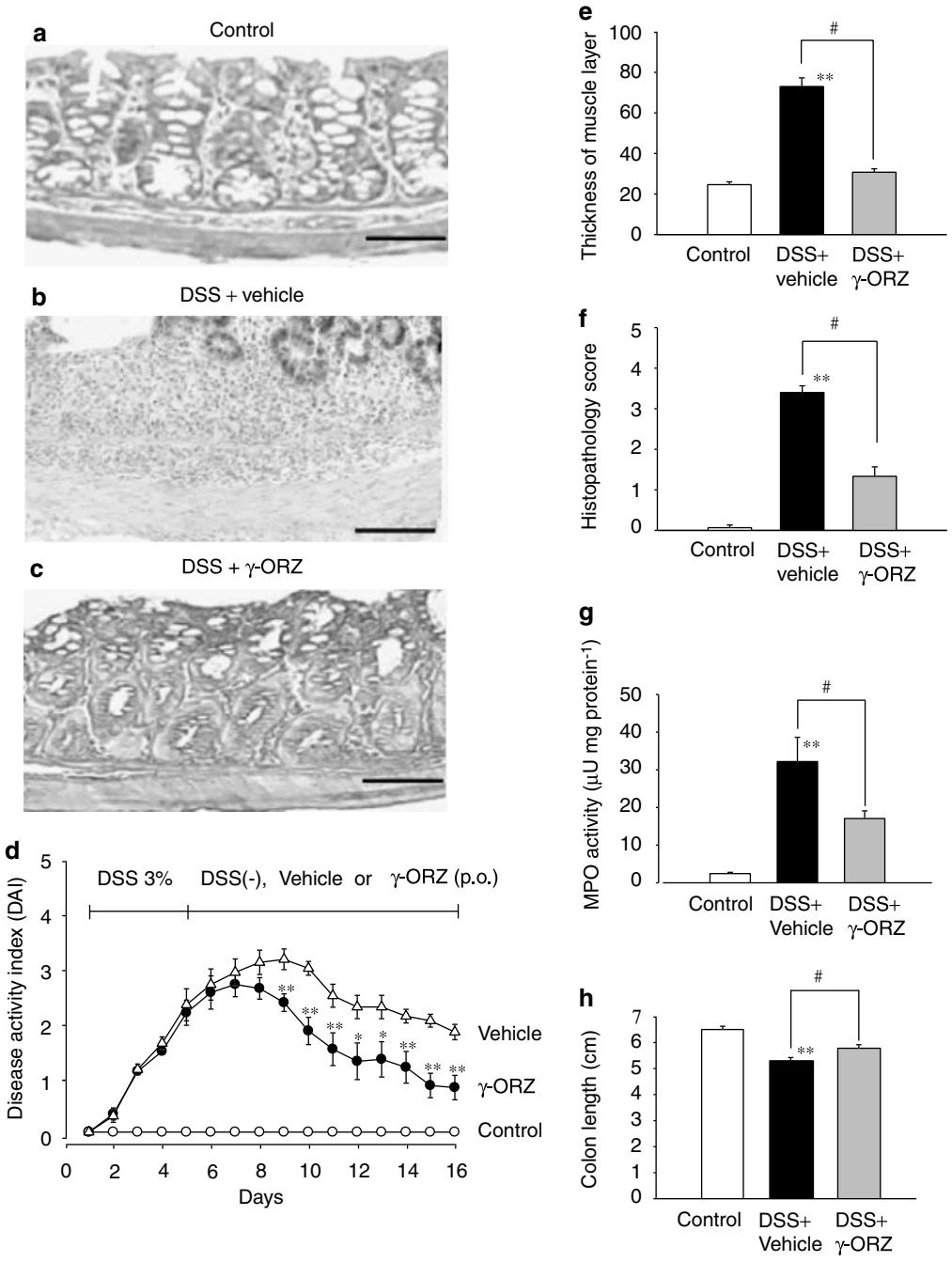

Figure 9 Therapeutic effect of γ-ORZ in DSS-induced colitis mice. Experimental colitis was induced by 3% DSS for 5 days. After induction of colitis, γ-ORZ (50 mg kg^{-1} day^{-1} p.o.) or vehicle was administered orally, commencing at the sixth day, up to day 16. Histology (haematoxylin and eosin staining) of full thickness of mid-colon are shown in (**a**) non-treatment:control, (**b**) DSS + vehicle and (**c**) DSS + γ-ORZ. (**d**) DAI (*$P<0.05$; **$P<0.01$, significantly different from DSS treatment), (**e**) Thickness of muscle layer, (**f**) histopathology score, (**g**) colonic MPO activity and (**h**) colon length (details described in the Materials and methods). **Significantly different from control ($P<0.01$). #Significantly different between DSS + vehicle and DSS + γ-ORZ treatment ($P<0.05$). Values are expressed as mean ± s.e.mean. $n=5$. Bar = 50 μm. DAI, disease activity index; DSS, dextran sulphate sodium; MPO, myeloperoxidase; γ-ORZ, γ-oryzanol.

study, we selected CAF and evaluated its anti-inflammatory properties. As demonstrated in Figure 7, CAF significantly inhibited DAI, histopathology score and MPO activity. A large number of experimental and clinical data suggests that chronic gut inflammation may be the result of a sustained overproduction of reactive metabolites of oxygen and nitrogen (Pavlick *et al.*, 2002). Therefore, there is a possibility that FA, a possible metabolite of γ-ORZ, would simultaneously change the oxidative and inflammatory variables in DSS-induced inflammation, *in vivo*. Indeed, sodium ferulate significantly reduced inducible NOS, COX-2 and NF-κB p65 protein expression in colonic

mucosa of rats with colitis (Dong *et al*., 2003). In the present study, we also demonstrated that FA weakly but significantly inhibited inflammatory parameters of DSS-induced colitis (Figure 7).

One of the major transcription factors involved in the pro-inflammatory gene regulation is NF-κB. NF-κB is generally considered to be present in the cytoplasm as a heterodimer complex of p65/p50 subunits combined with an inhibitory protein, IκB. Inflammatory stimuli induce rapid degradation of IκB, and subsequently the free NF-κB molecule translocates into the nucleus, binds to target DNA elements and activates transcription of genes encoding proteins involved with inflammation responses (Baldwin, 1996; May and Ghosh, 1998). Recently, we reported that phytosteryl ferulates significantly inhibited inducible NOS expression and NO production in activated macrophages by interfering with NF-κB activation (Nagasaka *et al*., 2007). In the present study, we examined nuclear translocation of NF-κB p65 subunit *in vivo*. As demonstrated in Figure 5, NF-κB p65 was stained mainly in the nuclei of epithelial cells as well as inflammatory cells in DSS-treated mice. However, in DSS + γ-ORZ-treated mice, NF-κB p65 was stained in the cytoplasm of the epithelial cells, and nuclear staining of NF-κB p65 was weak. Western blot analysis further confirmed this result; that is, the nuclear component of NF-κB p65 was significantly increased in the DSS-treated mice, which was reduced by γ-ORZ treatment. These results suggest that the inhibitory effects of γ-ORZ on the development of DSS-induced colitis were associated with the blockade of NF-κB activation.

The question then arises how γ-ORZ exerts its anti-inflammatory effects on DSS-induced colitis through NF-κB mediated reactions. It is well documented that reactive oxygen species activates NF-κB, which leads to the generation of proinflammatory cytokines and inducible enzymes, such as COX-2 and inducible NOS, in leukocytes and macrophages. Conversely, the proinflammatory cytokines caused oxidative stress by promoting the release of reactive oxygen species by immune and non-immune cells. Thus, inflammation and oxidative stress are involved in the spiraling vicious cycle that contributes to the severity of the intestinal inflammation (Reifen *et al*., 2004). In the present study, we demonstrated that γ-ORZ and one of its components, CAF, exhibited strong antioxidant activity compared to FA, a metabolite of γ-ORZ (Figure 8). These findings suggest that anti-inflammatory effect on DSS-induced experimental colitis may be mediated at least partly by the inhibition of NF-κB activity through scavenging reactions of free radicals. The role of reactive oxygen species in initiating and controlling the phosphorylation cascades that result in NF-κB activation is still controversial; however, several lines of evidence suggest that application of oxidative stress such as H_2O_2 to cells does stimulate IκB degradation and that addition of antioxidants prevents these phenomena (D'Angio and Finkelstein, 2000). One important finding of our study was that reduced immunostaining for IκB-α was observed in DSS-treated mice and this was increased by γ-ORZ treatment (Figure 6). From these results, it may be suggested that the oral administration of γ-ORZ prevents degradation of IκB, thereby inhibiting nuclear localization of NF-κB, which results in an amelioration of DSS-induced colitis.

An alternative possibility is that γ-ORZ exerts anti-inflammatory activity not only by scavenging reactive oxygen species but also inducing *de novo* expression of genes that encode defensive proteins, such as glutathione peroxidase, haem oxygenase-1 and nicotinamide adenine dinucleotide phosphate/quinone reductase (Na and Surh, 2006). It should also be taken into account that NF-κB is directly regulated by redox reactions through the modification of conserved cysteine residues that are located in the DNA-binding domain (Kamata and Hirata, 1999). The redox-sensitive mechanism responsible for the regulation of NF-κB, however, is still not fully understood, and further studies are necessary to solve the precise mechanism.

Curcumin (diferuloylmethane), the yellow pigment of turmeric in curry, has received much attention as a promising dietary supplement for the prevention and treatment of cancer (Maheshwari *et al*., 2006; Jagetia and Aggarwal, 2007). Recently, curcumin has been shown to cause apoptosis of multiple myeloma cells via inhibition of NF-κB and STAT3 activation (Bharti *et al*., 2003). Curcumin has also been shown to attenuate chronic colitis by decreasing the expression of COX-2 and increasing prostaglandin E_2 (Jiang *et al*., 2006; Camacho-Barquero *et al*., 2007). Although the exact mechanism for the ameliorative effects of curcumin and phytosteryl ferulates on intestinal inflammation is not known, these natural compounds warrant further consideration as a potential immunoregulatory treatment in clinical IBD.

In conclusion, this study has demonstrated that anti-inflammatory effects in DSS-induced experimental colitis may be mediated by the inhibition of NF-κB activity, which is at least partly reflects the antioxidant effect of the FA moiety in the phytosteryl ferulate molecule. These results strongly suggest that modulation of pathophysiological activity during colonic inflammation by phytosteryl ferulates might be a promising therapeutic means for the treatment of diseases characterized by inflammation of the gastrointestinal tract. Current medical therapy in IBD still involves the administration of aminosalicylates, glucocorticoids and immunomodulators. A comparative assessment including the above-mentioned agents and phytosteryl ferulates should also be evaluated in a future study.

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Conflict of interest

The authors state no conflict of interest.

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