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RESEARCH PAPER

Protection from experimental colitis by theaflavin-3,3′-digallate correlates with inhibition of IKK and NF-κB activation

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Background and purpose: Inflammatory bowel disease (IBD) is associated with activation of nuclear factor kappa B (NF-κB) involved in regulating the expression of inducible nitric oxide synthase (iNOS) and proinflammatory cytokine genes. As theaflavin-3,3′-digallate (TFDG), the most potent anti-oxidant polyphenol of black tea, down-regulates NF-κB activation, we investigated if TFDG is beneficial in colonic inflammation by suppressing iNOS and proinflammatory cytokines.
Experimental approach: The *in vivo* efficacy of TFDG was assessed in mice with trinitrobenzene sulfonic acid (TNBS)-induced colitis. Both mRNA and protein levels of proinflammatory cytokines and iNOS were analyzed in colon tissue treated with or without TFDG. NF-κB activation was determined by electrophoretic mobility shift assay and levels of NF-κB inhibitory protein (IκBα) were analyzed by Western blotting.
Key results: Oral administration of TFDG (5 mg kg^{-1} daily i.g.) significantly improved TNBS-induced colitis associated with decreased mRNA and protein levels of TNF-α, IL-12, IFN-γ and iNOS in colonic mucosa. DNA binding and Western blotting revealed increase in NF-κB activation and IκBα depletion in TNBS-treated mice from Day 2 through Day 8 with a maximum at Day 4, which resulted from increased phosphorylation of IκBα and higher activity of IκB kinase (IKK). Pretreatment with TFDG markedly inhibited TNBS-induced increases in nuclear localization of NF-κB, cytosolic IKK activity and preserved IκBα in colon tissue.
Conclusions and Implications: TFDG exerts protective effects in experimental colitis and inhibits production of inflammatory mediators through a mechanism that, at least in part, involves inhibition of NF-κB activation.
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Keywords: inflammatory bowel disease; theaflavin-3-3′-digallate; TNBS-induced colitis; nuclear factor κB; proinflammatory cytokine; inducible nitric oxide synthase

Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa B; IκBα, NF-κB inhibitory protein; IKK, IκB kinase; TFDG, theaflavin-3,3′-digallate; TFD1, theaflavin-3-gallate; TFD2, theaflavin-3′-gallate; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TR, thearubigin; UC, ulcerative colitis

Introduction

Inflammatory bowel disease (IBD), comprising two major disease entities, Crohn's disease (CD) and ulcerative colitis (UC) and identifiable by a set of clinical, endoscopic and histological features (Kirsner and Shorter, 1988) is of still unknown aetiology. In most of the chronic inflammatory diseases, adhesion molecules recruit inflammatory cells, such as neutrophils, eosinophils and T lymphocytes, from the circulation to the site of inflammation (Albelda *et al*., 1994). In a majority of animal models of intestinal inflammation, including the well-characterized hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model, increased levels of mucosal proinflammatory cytokines such as IL-1β, IL-6, TNF-α, IL-12 and IFN-γ have been shown to play an important role in sustained inflammatory responses (Elson *et al*., 1995; Strober *et al*., 1998; Boismenu and Chen, 2000). Expression of these immunomodulatory proteins appear to be regulated mainly by the transcription factor NF-κB (Baldwin, 1996). In most cells, NF-κB, a predominant heterodimer consisting of p65 (RelA) and p50 subunits, is present as a latent, inactive, IκB bound complex in the cytoplasm but upon activation by extracellular stimuli or by other factors, NF-κB rapidly

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translocates to the nucleus and activates gene expression (May and Ghosh, 1998). The exact molecular mechanism by which various extracellular stimuli lead to the activation of NF-κB is not well understood. However, most signals induce the activity of a large multisubunit protein kinase, called IκB kinase (IKK). Activation of the IKK complex leads to specific IκBα phosphorylation/degradation and subsequent release of NF-κB, which then translocates to the nucleus and activates transcription of multiple κB-dependent genes including iNOS and Th1 cytokines. NF-κB has already been shown to be strongly activated in UC and CD (Neurath *et al*., 1998; Rogler *et al*., 1998; Schreiber *et al*., 1998). Experimental trials using p65 antisense oligonucleotides against NF-κB in TNBS-induced colitis model efficiently prevent NF-κB activation and cytokine production resulting in the prevention of mucosal inflammation (Neurath *et al*., 1996; Hamamoto *et al*., 1999). Moreover, compounds which downregulate NF-κB have been proved to be beneficial in the treatment of IBD (Sugimoto *et al*., 2002).

Medications currently available for IBD alleviate inflammation and reduce symptoms, but do not provide a cure or prevent long-term complications. The principal drugs used are 5-aminosalicylate, corticosteroids and immunosuppressants. Although these drugs have shown their benefit in the treatment of IBD, they have serious side effects that limit their clinical application. Promising treatments that might be considered as adjuncts to conventional treatment include herbal medicines derived from plant extracts that are being utilized to treat a wide variety of clinical diseases. Recently, there has been a great scientific interest in the field of phytochemicals, especially tea, as nutritional tools for chronic disease prevention, a definitive approach to control disease and reach old age in good health. Tea is consumed worldwide as black tea, oolong tea and green tea. Although black tea is the most widely consumed (80% of the total tea consumption) beverage, the work carried out on black tea so far is much less compared to green tea. Tea polyphenols especially catechins of green tea, have been the primary agent responsible for the beneficial and disease-inhibitory activity (Dufresne and Farnworth, 2001). During the production of black tea, substantial proportions of catechins are converted to theaflavins and thearubigins by a polyphenol oxidase (Balentine *et al*., 1997). Theaflavins (about 1–2% of the total dry weight of black tea) including theaflavin, theaflavin-3-gallate (TFD1), theaflavin-3′-gallate (TFD2) and theaflavin-3,3′-digallate (TFDG), possess benzotropolone rings with dihydroxy or trihydroxy systems. Only recently work has been initiated with black tea or its characteristic constituents, theaflavins and thearubigins (TR), which have revealed diverse pharmacotherapeutic effects including hypoglycemic (Gomes *et al*., 1995), anticarcinogenic (Mukhtar and Ahmad, 2000; Schwab *et al*., 2000) and antiatherosclerotic (Muramatsu *et al*., 1986) effects. A number of studies have shown that tea polyphenols especially TFDG, inhibit production of NO and expression of iNOS mRNA by murine macrophages (Chan *et al*., 1997; Kim *et al*., 1999; Lin *et al*., 1999) and this TFDG mediated inhibition of iNOS is associated with downregulation of NF-κB activation (Lin *et al*., 1999). These observations suggest that TFDG may be useful in the treatment of patients with IBD.

In the present, study we administered TFDG to mice with TNBS-induced colitis to evaluate its beneficial effects on intestinal inflammation. Our results demonstrate that treatment with TFDG depends on its ability to suppress the TNBS-induced activation of NF-κB and increased production of proinflammatory cytokines such as IL-12, IFN-γ and TNF-α.

Methods

Animals

Female BALB/c mice weighing 25–30 g (obtained from National Institute of Nutrition, Hyderabad, India) were used for the experiments. Mice were housed under normal laboratory conditions that is at 21–24°C and 40–60% relative humidity, under a 12 h light/dark cycle with free access to standard rodent food and water. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23 revised 1996) and with the approval of the Institutional Animal Care and Use Committee.

Experimental colitis

To induce colitis, mice were lightly anesthetized with diethyl ether and 0.1 ml of TNBS (60 mg ml^{-1} in 30% ethanol), was slowly administered into the lumen of the colon through a trochar needle approximately 3–4 cm proximal to the anus according to the model described earlier (Neurath *et al*., 1995). Control group of mice received 30% ethanol in PBS using the same technique. Animals were killed at 2, 4 and 8 days after TNBS administration. TFDG was administered by oral gavage in 200 μl of PBS (freshly prepared) every day, over a 18-day period. To investigate the dosage effect, 1, 2.5, 5 and 10 mg kg^{-1} of TFDG was given daily by oral gavage for 10 days before subjecting the mice to TNBS-induced colitis and the same dose of TFDG was continued until the mice were killed 8 days after the induction of colitis (Figure 1). Five or six animals were used for each time point after TNBS administration.

Macroscopic assessment of severity of colitis

Mice were killed by cervical dislocation, the colon excised, opened longitudinally and washed in saline. Macroscopic damage was assessed by the scoring system of Wallace and Keenan (1990), which takes into account the area of inflammation and the presence and absence of ulcers. The criteria for assessing macroscopic damage was based on a semi-quantitative scoring system where features were graded as follows: 0, no ulcer, no inflammation; 1, no ulcer, local hyperaemia; 2, ulceration without hyperaemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation and 5, ulceration extending more than 2 cm. After macroscopic observation, samples of colonic tissue were subsequently excised for microscopic observation of damage, measurement of NO and mRNA expression of cytokines and iNOS.

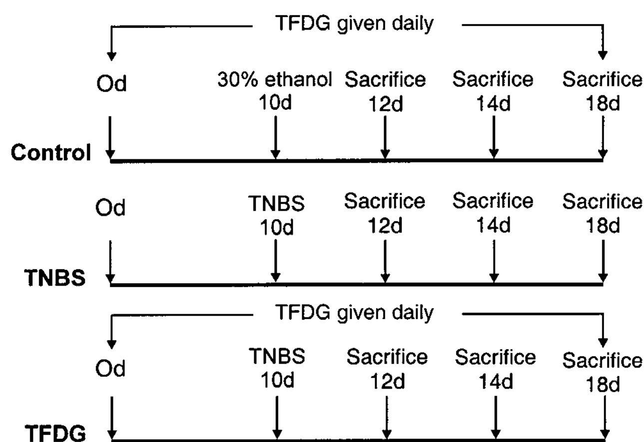

Figure 1 Time course of experimental schedules. Control and TFDG group received TFDG daily by oral gavage for a total of 18 days. Both TNBS and TFDG group received TNBS by intracolonic administration at day 10 whereas the control group received 30% ethanol at day 10. Groups of mice were killed (Sample) at 12, 14 or 18 days after the start of the study.

Microscopic assessment of colitis
The colon was fixed in 10% formalin in *PBS* for 1 week and the samples were then dehydrated in graded ethanol and embedded in paraffin. Sections 7 μm were deparaffinized with xylene, stained with hematoxylin–eosin and examined in a Leitz Ortholux microscope. Histologic changes were graded semi quantitatively from 0 to 4 according to previously described criteria (Neurath *et al*., 1995) as follows: 0, no leukocyte infiltration; 1, low level of leukocyte infiltration; 2, moderate level of leukocyte infiltration; 3, high vascular density and thickening of colon wall and 4, transmural leukocyte infiltration, loss of goblet cells, high vascular density and thickening of the colon wall.

Macrophages
Macrophages were collected by peritoneal lavage from mice (BALB/c; 20–25 g) given intraperitoneal (i.p.). injection of 0.5 ml of 4% thioglycollate broth 5 days before harvest and were utilized as described earlier (Basu *et al*., 1991). The culture medium consists of RPMI 1640 supplemented with 10 mM HEPES, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% FCS. More than 90% of the cell preparation was identified as macrophages by microscopic observation, and the macrophages were routinely found to be >95% viable by Trypan blue exclusion.

NO production
Nitrite production was determined in macrophage culture medium by the Griess reaction as previously described (Das *et al*., 2001). Cell viability was assessed using a MTT-based colorimetric assay kit (Roche Applied Science, Basel, Switzerland) according to manufacturer's instructions.

Myeloperoxidase activity
Measurement of myeloperoxidase (MPO) activity is used as a biochemical marker of neutrophil infiltration into gastrointestinal tissues (Morris *et al*., 1989). The MPO activity of the tissue was determined by the method described earlier (Ukil *et al*., 2003).

Analysis of cytokines
The colon was homogenized in ice-cold tissue protein extraction medium (Pierce, Rockford, IL, USA) containing PMSF (10 μg ml^{-1}), aprotinin (10 μg ml^{-1}), leupeptin (10 μg ml^{-1}), *N*-p-tosyl-L-lysine chloromethyl ketone (10 μg ml^{-1}) and L-1-tosylamido-2-phenylethyl chloromethyl ketone (10 μg ml^{-1}). The homogenate was then centrifuged at 18 000 *g* for 20 min at 4°C. IFN-γ, IL-12 and TNF-α were measured in the supernatant using an ELISA kit (BD Biosciences, San Jose, CA, USA). mRNA profiles for these cytokines along with β-actin as internal control were analyzed by RT-PCR. Reverse transcription of 1 μg of RNA was performed according to the manufacturer's protocol for the Superscript One-Step RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA, USA). Primers for all these genes have been published (Kawakami *et al*., 1997). After the appropriate number of PCR cycles, the amplified cDNA was separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

iNOS expression by RT-PCR and immunoblot analysis
RT-PCR was performed to determine the mRNA profile for iNOS along with β-actin as internal control. For immunoblot analysis, 20 μg of colonic extracts were resolved by SDS-PAGE, electroblotted to nitrocellulose membrane and probed with murine anti-iNOS antibody (Transduction Laboratories, Lexington, KY, USA). After extensive washing, the antigen-antibody complexes were detected by the ECL kit (Amersham Biosciences, Arlington Heights, IL, USA) using horseradish peroxidase-conjugated anti-rabbit IgG (1:2000 dilution).

Preparation of nuclear and cytoplasmic extracts
A modified procedure based on Yang *et al*. (1998) was used. Briefly, the excised colons were washed with PBS (pH 7.4) and homogenized in ice-cold lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 0.5 mM dithiothreitol and 1 μg ml^{-1} of aprotinin, leupeptin and pepstatin A), using a Dounce homogenizer. Homogenates were then incubated on ice for 45 min, NP-40 was added to a final concentration of 0.5% and the mixture was centrifuged at 5000 *g* for 10 min at 4°C. The supernatant was saved for analysis of cytosolic proteins. The pellet was extracted in nuclear extraction buffer (20 mM HEPES, pH 7.8, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, 0.5 mM DTT and 1 μg ml^{-1}of aprotinin, leupeptin and pepstatin A) for 30 min on ice and centrifuged at 15 000 *g* for 15 min. Protein concentrations were determined using Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA). Extracts were stored at −80°C.

Electrophoretic mobility shift assay

For electrophoretic mobility shift assay (EMSA), each 10 μg of nuclear extracts were preincubated with 1 μg of poly (dI-dC) in a binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, 5% glycerol and 50 mM NaCl) for 10 min at room temperature. As a control, a 50-fold molar excess of unlabeled NF-κB competitor oligonucleotide was added. After preincubation, 0.5 ng of ^{32}P end-labeled NF-κB oligonucleotide probe (5′-CGGGGACTTT CCGCTGGGGACTTTCCGCTTGAGCT-3′) was added to the reaction mixture and incubated for 30 min. The DNA-protein complex was then electrophoresed on 4.5% nondenaturing polyacrylamide gels in 0.5 × TBE buffer (0.0445 M Tris, 0.0445 M borate and 0.001 M EDTA). For supershift assay, the nuclear extracts from TNBS-treated cells were incubated with antibodies against individual components of NF-κB complex (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at 25°C and analyzed by EMSA in the presence of all components of the binding reaction described above. As controls, the nuclear extracts were also treated with normal rabbit IgG.

Western blot analysis of IκBα, p65 and p50

The nuclear and cytosolic proteins (30 μg) prepared from mice colon were resolved by 12% SDS-PAGE and electrotransferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). Membranes were washed with Tris-buffered saline (TBS) (100 mM Tris-HCl, 0.9% NaCl, pH 7.5) and immunoblotted as follows. Nonspecific binding sites were blocked with TBS containing 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk at room temperature for 18 h. Membranes were then incubated in a 1:1000 dilution of rabbit polyclonal anti-IκBα for cytosolic proteins or anti-p50 and anti-p65 antibodies for nuclear proteins (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBS-T. After four washes in TBS-T, membranes were incubated in a 1:5000 dilution of horseradish peroxidase-conjugate anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. After washing, immunoreactive proteins were detected by enhanced chemiluminescence using an ECL detection kit (Amersham Biosciences, Arlington Heights, IL, USA). Band intensities were quantitated densitometrically using QUANTITY ONE (Bio-Rad, Hercules, CA, USA).

IKK assay

The assay was performed as described by Andresen *et al*. (2005). 300 μg of cytosol extract were immunoprecipitated with anti-IKKα/β antibody in immunoprecipitation buffer (40 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM pNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin, 1 mM DTT and 0.1% Nonidet P-40). Immunoprecipitated samples were incubated with recombinant IκBα (4 μg) in kinase buffer (20 mM HEPES, pH 7.7, 2 mM $MgCl_2$, 10 μM ATP, 3 μCi of [γ^{32}-p]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM pNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin and 1 mM DTT) at 30°C for 1 h. The kinase reaction was stopped by addition of SDS-sample buffer. The sample was resolved by SDS-PAGE, dried and autoradiographed. To determine the total amounts of IKKα and IKKβ in each sample, 30 μg of the whole cell extract protein was subjected to SDS-PAGE and analyzed by Western blot using anti-IKKα and IKKβ antibody.

Statistical analysis

Results are expressed as mean ± s.d. of n observations. We used analysis of variance to determine the statistical significance of inter group comparisons. $P < 0.05$ was considered to be statistically significant. Macroscopic and microscopic scores for colonic erosions for the TFDG-pretreated groups were compared with those for the TNBS-treated group with a two-sided Wilcoxon rank-sum test.

Materials

TFDG, TFD1, TFD2 and TR were isolated from CTC (curl, tear and crush) BOP (broken orange pickoe) grade black clonal tea processed in October 1991 and supplied by Tocklai Experimental Station, Jorhat, Assam, India (collection No. 01091) according to the method described earlier (Chen and Ho, 1995). The purity of TFDG was determined to be >98% by HPLC. TNBS and all other chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA). Rabbit polyclonal antibodies for NF-κB p65, p50, IκBα, IKKα and IKKβ and NF-κB consensus oligonucleotide and recombinant IκBα were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Results

Inhibition of NO generation by tea polyphenols

IBD and animal models of colitis are characterized by high levels of NO generation by iNOS, which can result in mucosal injury (Sandborn and Yednock, 2003). As TFDG might act as a therapeutic agent for IBD through inhibition of NO production, we first investigated the ability of TFDG to downregulate NO in LPS-activated murine macrophages. As shown in Figure 2a, TFDG could downregulate NO production in peritoneal macrophages in a concentration-dependent manner. The effect was maximal with a concentration of 40 μM after incubation for 24 h. TFDG exhibited no cytotoxicity at concentrations up to 50 μM. Furthermore, when selected polyphenols of black tea were tested for inhibition of NO, TFDG was found to be the most effective (Figure 2b), which is in agreement with the earlier observation by Lin *et al*. (1999). The level of inhibition by various polyphenols is also consistent with the expression of iNOS mRNA, which, after isolation of total RNA, was subjected to RT-PCR analysis (Figure 2c).

Effect of TFDG on TNBS-induced colitis

As TFDG could downregulate NO production together with the absence of obvious cytotoxicity on macrophages, we

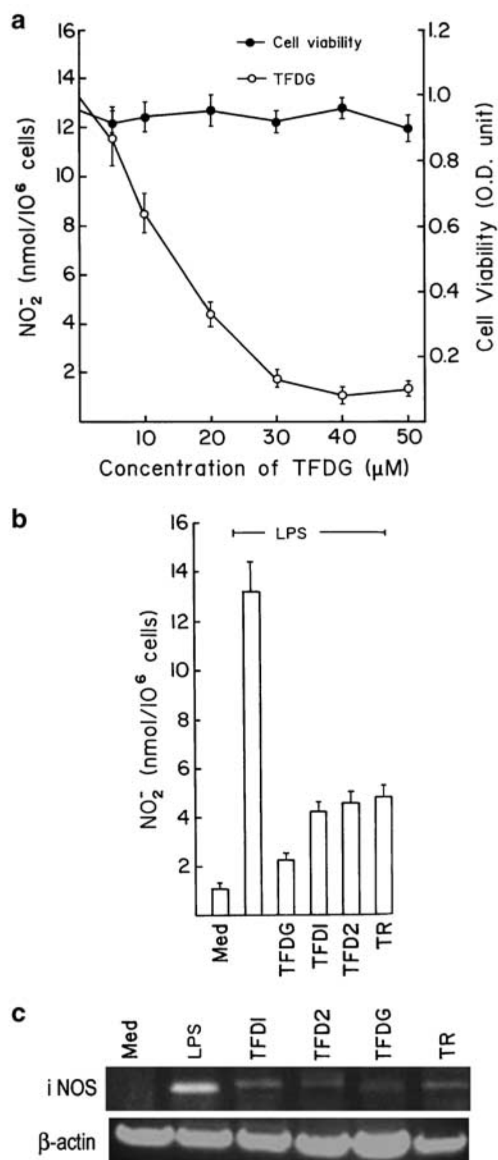

Figure 2 Effect of TFDG on NO production. (**a**) Peritoneal macrophages (10^6 cells ml^{-1}) were cotreated with LPS (100 ng ml^{-1}) and various concentrations of TFDG for 24 h at 37°C. Cell viability was assessed by an MTT assay. (**b**) Macrophages (10^6 cells ml^{-1}) were incubated with LPS (100 ng ml^{-1}) either alone or in the presence of 40 μM each of TFDG, TFD1, TFD2 or TR. Each bar shows the mean $\pm$ s.d. of three independent experiments. (**c**) The nature of iNOS expression by various agents was also determined by RT-PCR of its mRNA transcript.

checked the efficacy of TFDG on TNBS-induced colitis. Single intracolonic administration of 6 mg TNBS in 30% ethanol reproducibly induced a transient IBD-like colitis with maximum inflammation at 4 days. Significant edema formation along with focal ulcerations, necrosis and adhesions was observed in the descending colon of the TNBS control group. The dose titration experiment assessed the efficacy of TFDG against TNBS-colitis with a dose range of 1–10 mg ml^{-1} given daily for 10 days before induction of colitis as well as during the course of the disease. Oral administration of TFDG significantly reduced the extent and severity of the injury of large intestine in a dose-dependent manner as shown by both macroscopic and microscopic damage scores (Figure 3a and e). As expected, TFDG significantly reduced colonic MPO activity, an index of neutrophilic infiltration in the mucosa (Figure 3f). TFDG pretreatment also significantly reversed the decrease of body weight and increase of spleen and colon weight, an indicator of inflammation, associated with TNBS-induced colitis (Figure 3b, c and d). The results indicate that 5 mg kg^{-1} day^{-1} might be regarded as the lowest active dose of TFDG for effective treatment. Histochemistry demonstrated prominent infiltration of granulocytes into the affected colon of TNBS control, especially in the most severely damaged regions (Figure 3h) at day 4. In contrast, in colonic sections from TFDG-treated mice (5 mg kg^{-1} day^{-1}), only a few granulocytes were observed in the mucosa and submucosa propria (Figure 3i). Regarding local effects of TFDG in the colons, control experiments were carried out using TFDG pretreatment in the absence of TNBS treatment as well as TFDG pretreatment in 30% ethanol. In both the controls, no histological modifications were observed as compared to control mice receiving 30% ethanol.

Effects of TFDG on cytokine mRNA levels

To evaluate the type of immunological response in TNBS-induced colitis due to TFDG treatment, mRNA transcription levels for TNF-α, IFN-γ and IL-12 p40 were determined on colonic mucosal cells. The increases in all the cytokine mRNA levels occurred in TNBS-treated groups from day 2 through day 8 but most significant increases were seen at day 4 compared with control (ethanol-treated) at corresponding times (Figure 4a). This represented a dominant inflammatory Th1 response in TNBS-induced colitis. However, pretreatment with TFDG (5 mg kg^{-1} day^{-1}) significantly decreased the message levels in TNBS-treated groups in all the time points from day 2 through day 8 compared with TNBS-treated group at corresponding time (Figure 4a). For additional confirmation, cytokine production was assessed in colonic mucosal cells of TFDG-treated animals at the protein level by ELISA, which showed significantly less TNF-α, IFN-γ and IL-12 p40 than those from TNBS control (Figure 4b). TFDG pretreatment also resulted in downregulation of iNOS in TNBS-group at both mRNA and protein level (Figure 5). These results not only suggest suppression of Th1 functions but also show the potential of TFDG as a proper effector molecule, which might induce protective responses.

Effect of TFDG on NF-κB DNA binding and activation

The apparent decrease of iNOS and Th1 cytokine profile by TFDG led us to examine the status of NF-κB in control and treated mice, because this transcription factor is known to regulate the production of various proinflammatory cytokines such as IL-12, IL-1β and TNF-α as well as the activation of specific enzymes such as iNOS and cyclooxygenase. The kinetics of NF-κB activation in colonic mucosa groups were determined by EMSA using nuclear extracts of whole colonic tissues from control mice (which received 30% ethanol without TNBS) and TFDG-treated or untreated TNBS colitis

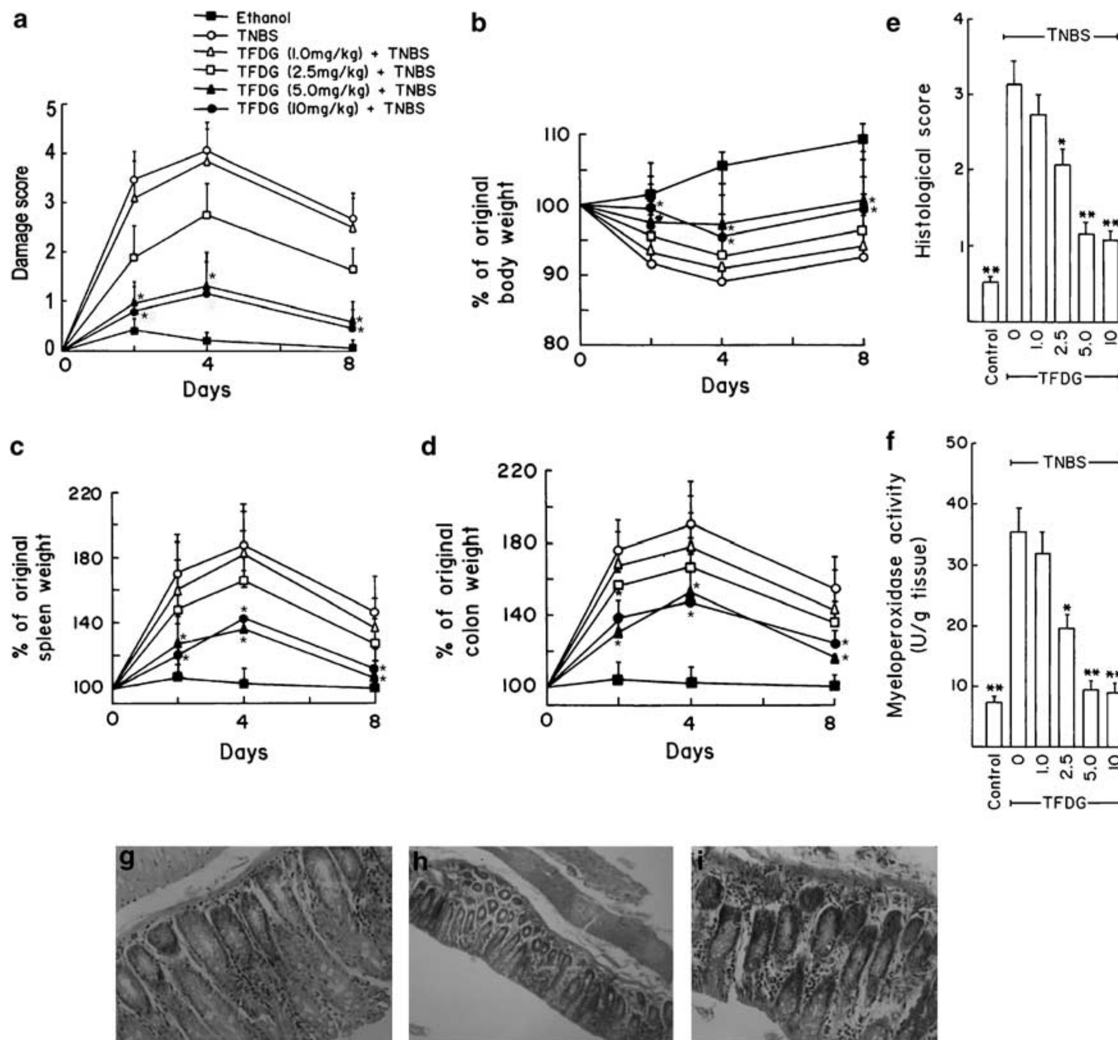

Figure 3 Effect of TFDG treatment on established colitis. Mice were treated with 0.1 ml of TNBS (60 mg ml^{-1}) intracolonically and assessed at various times (2–8 days) after treatment. (**a**) Colonic damage was scored by blinded assessment based on validated scoring system as described in Methods. Evaluation of body weights (**b**), spleen (**c**) and colon (**d**) expressed as a percentage of the original weight on day 0. A significant weight gain was observed at 4 days after TNBS administration in spleen and colon. TFDG pretreatment (5 and 10 mg kg^{-1}) significantly prevented the loss in body weight (**b**) as well as reduced the organ weight (**c** and **d**). (**e**) Histological scoring was performed semi-quantitatively in hematoxylin and eosin-stained sections. (**f**) Assessment of myeloperoxidase activity in TNBS-treated mice in comparison to control. Values are means ± s.d. of 10 mice for each group. Results for the TFDG treatment group were compared against those for the TNBS-treated group with a two-sided Wilcoxon rank-sum test. **P*<0.01, ***P*<0.05 vs TNBS. Histological appearance of mice colonic mucosa after hematoxylin and eosin stain, treated with 30% ethanol in PBS (**g**), treated with TNBS (60 mg ml^{-1}) in 30% ethanol (**h**) and pretreated with TFDG (5 mg kg^{-1}) (**i**). TNBS-induced colonic inflammation (at 4 days) was mostly associated with loss of goblet cells, crypt damage, mucosal ulceration and accompanying edema of the submucosa. Pretreatment with TFDG significantly attenuated the damages in morphology associated with TNBS treatment. Original magnification: ×250.

mice (Figure 6a). A basal level of NF-κB DNA-binding activity was found in colonic nuclear extracts from mice in control group. The nuclear localization of NF-κB in the TNBS-treated group was increased after day 2 compared to control group, became maximal at day 4 and again decreased at day 8. TNBS-induced enhancement of NF-κB DNA-binding activity was suppressed by pretreatment with 5 mg kg^{-1} day^{-1} TFDG at all time points. Excess unlabeled specific oligonucleotides inhibited the NF-κB mobility shift, indicating thereby the specificity of DNA-protein complex. To identify the specific NF-κB subunits that comprise the NF-κB signal detected by EMSA in TNBS-induced colitis at day 4, supershift assay was performed. Specific antibodies to p50, p52, p65, c-Rel and Rel B were used for these experiments. Supershift studies demonstrated that antibodies to p65 shifted the entire signal and that antibodies to p50 also caused a significant shift. However, anti-p52, anti-c-Rel or anti-Rel B antibodies did not shift the NF-κB signals (Figure 6b). To determine the effect of TFDG on the migration of p50 and p65 into the nucleus, Western blot analyses of nuclear protein fractions from colonic mucosa were conducted. Accumulation of p50 and p65 subunits in the nuclear protein fraction was greatly increased in TNBS-treated mice at all time points examined (Figure 6c). However, consistent with the DNA-binding

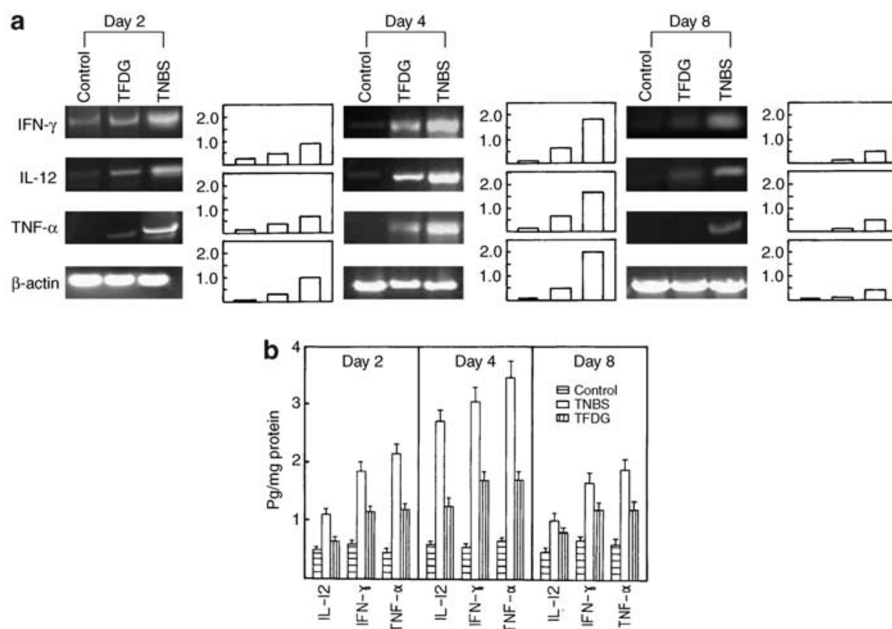

Figure 4 Proinflammatory cytokine expression in colonic mucosa of mice with TNBS-induced colitis. Oral pretreatment with TFDG ($5\,mg\,kg^{-1}$) showed a significant decrease in the expression levels of IFN-γ, IL-12 p40 and TNF-α in the colon of TNBS-treated mice both at mRNA (**a**) and protein (**b**) level as analyzed by RT-PCR and ELISA, respectively. RT-PCR products were visualized by ethidium staining. RNA samples were obtained from six mice in each group. Results are representative of three separate samples. β-actin expression levels were used as controls for RNA content and integrity. Band intensities quantified by densitometry are shown as bar diagrams.

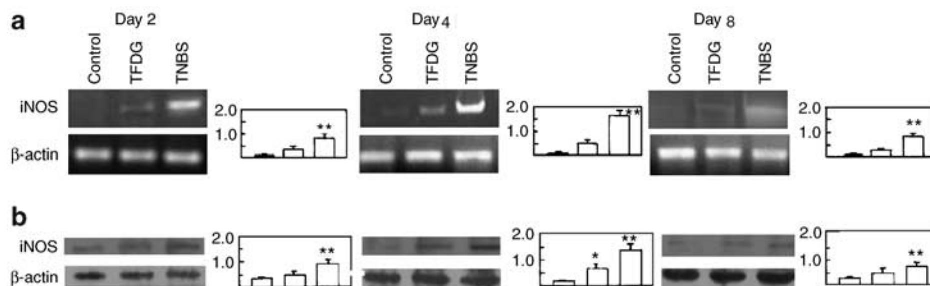

Figure 5 A time–course pattern of iNOS expression. iNOS expression by RT-PCR (**a**) and Western blot (**b**) in colonic mucosa of colitic mice and TFDG-pretreated colitic mice on day 2, 4 and 8 after TNBS administration. RT-PCR products were visualized by ethidium bromide staining. RNA samples were obtained from six mice in each group. Results are representative of duplicate samples of three separate experiments and the densitometric evaluations are means of three independent experiments. *$P<0.01$; **$P<0.001$ vs control. β-actin expression levels were used as controls for RNA content and integrity.

activity, nuclear translocation of both p50 and p65 subunits, which peaked at day 4 after TNBS-treatment, were significantly suppressed by oral administration of $5\,mg\,kg^{-1}\,day^{-1}$ TFDG.

Preservation of IκBα by TFDG pretreatment

The activation of NF-κB is thought to occur secondary to the proteolytic degradation of cytosolic IκBα, allowing free NF-κB to translocate to the nucleus where it binds to specific promoter sequences and initiates gene transcription (Henkel *et al*., 1993). As pretreatment with TFDG inhibited NF-κB activation in colonic mucosa of mice receiving TNBS, we determined if the protective effect of TFDG against TNBS-induced colon injury might be related to its effect in preserving cytosolic IκBα. Western blot analysis of the cytoplasmic extract prepared from colon homogenates revealed that IκBα protein levels were elevated in TFDG-treated group compared with untreated TNBS group (Figure 7a). Although a basal level of IκBα was there in the control group (30% ethanol), IκBα protein levels were greatly depleted in TNBS groups, indicating that NF-κB activation in this group occurred via IκBα degradation. In contrast, pretreatment with TFDG ($5\,mg\,kg^{-1}\,day^{-1}$) in TNBS groups protected IκBα degradation, which in turn prevented the translocation of NF-κB from the cytoplasm to nucleus. As IκBα is phosphorylated by the IKK multiprotein complex, the effect of TFDG on the status of intrinsic cellular IKK activation was determined. Accordingly, IKK was isolated from the colonic mucosal cytosolic extract of TFDG-treated

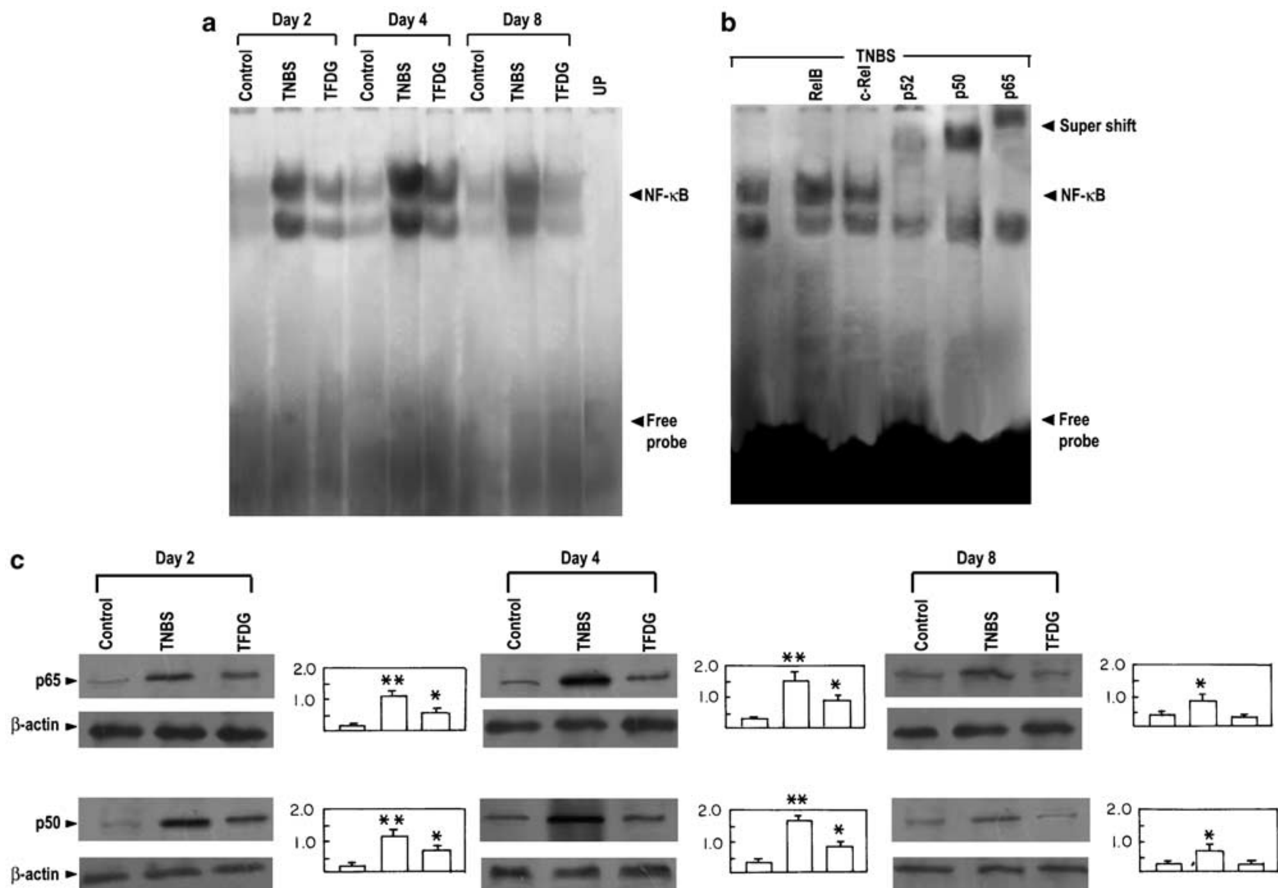

Figure 6 Effects of TFDG pretreatment (5 mg kg^{-1}) on the kinetics of NF-κB activation in colon tissue samples of mice with TNBS-induced colitis. (**a**) Nuclear extracts were obtained from mouse colon at the indicated time period and analyzed for NF-κB activity by EMSA. Each lane represents colon nuclear extracts from the control, TNBS or TFDG group. UP indicates 50× molar excess of unlabeled probe. Oral administration of TFDG consistently decreased the NF-κB-binding activity, which reached maximum at day 4 after TNBS administration and then started decreasing. (**b**) For supershift assay, nuclear extracts from colonic mucosa of mice at 4 day after TNBS administration were incubated with antibodies against individual components of NF-κB complex for 30 min before to EMSA analysis. (**c**) Western blot analysis of nuclear protein fraction shows a time-dependent migration of p50 and p65 into the nucleus of colon tissues in TNBS-induced colitis. Oral administration of TFDG (5 mg kg^{-1} day^{-1}) markedly suppressed the TNBS-induced nuclear transmigration. The blots were analyzed densitometrically and are normalized to β-actin. The results are representative of duplicate samples of three separate experiments and the densitometric evaluations are means of three independent experiments. *$P<0.01$; **$P<0.001$ vs control.

and untreated TNBS-colitic mice by immunoprecipitation with anti-IKKα and anti-IKKβ antibodies. The immunoprecipitated samples were used for kinase assay with recombinant IκBα as substrate. The radiolabeled phosphorylated IκBα-specific band was detected in mucosal cells of TNBS-treated mice, demonstrating the ability of TNBS to induce IKK activity (Figure 7b). In contrast, IKK activity was negligible in the normal untreated (30% ethanol) as well as TFDG-treated colitic mice (Figure 7b). Western blot analysis with whole cell lysates using anti-IKKα and anti-IKKβ antibodies (Figure 7b) showed identical levels of expression of IKK, suggesting that IKK was equally expressed in cells with different treatments. These results suggest that oral administration of TFDG prevented the TNBS-induced NF-κB activation by suppression of IKK activity resulting in preservation of the cytosolic IκBα protein. This may, in part, explain the reduced levels of proinflammatory cytokines in TNBS-treated groups after TFDG pretreatment.

Discussion and conclusions

Results of the present study have demonstrated that TFDG treatment could substantially reduce the TNBS-induced increased levels of inflammation and tissue injury in the mouse colon. This is consistent with the results reported by other investigators that the release of NO and proinflammatory cytokines play a central role in the TNBS-induced colitis. The ability of TFDG treatment to suppress the TNBS-induced release of these mediators, therefore, may constitute one of the possible mechanisms for its anticolitic effects. Further, the present study provides the first *in vivo* evidence that TFDG may exert its beneficial effects on experimental colitis at least in part, by inhibiting the IκBα-dependent signaling pathway.

Extensive experimental studies have shown that a variety of the pro-inflammatory mediators associated with IBD are regulated at the transcriptional level by the nuclear factor

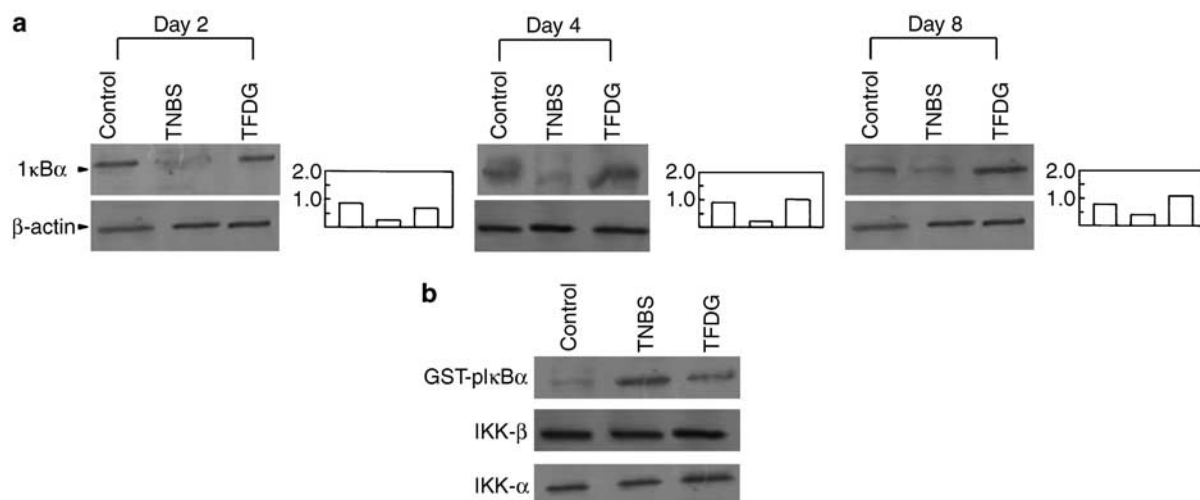

Figure 7 Effect of TFDG pretreatment on IκBα protein expression and IKK activation in the colon tissue of mice with TNBS-induced colitis. (**a**) Western blot analysis shows a time dependent increased preservation of IκBα protein in TFDG (5 mg kg^{-1} day^{-1}) pretreated groups as opposed to depletion in TNBS groups. The blots were analyzed densitometrically and the values are normalized to β-actin. Results are representative of one of three independent experiments. (**b**) Whole cell extracts were prepared and immunoprecipitated with antibodies against IKKβ and IKKα. Activity of immunoprecipitated IKK was measured using GST-IκBα as substrate and GST phosphorylated-IκBα was visualized by autoradiography. Relative amounts of IKKα and IKKβ in the whole cell extracts were determined by Western blots.

NF-κB and/or activator protein 1 (AP-1) (Karin *et al.*, 1997; Baeuerle, 1998). Increasing amounts of evidence are available to show that chronic inflammation of the intestine and colon can be ameliorated by the inhibition of NF-κB in experimental colitis (Togawa *et al.*, 2002). Inhibition of NF-κB in peripheral blood mononuclear cells and lamina propria mononuclear cells has been shown to offer an interesting alternative approach for the treatment of CD (Segain *et al.*, 2000). A few reports have also demonstrated a role for AP-1 activation in intestinal inflammation (Gonsky *et al.*, 1998; Abreu-Martin *et al.*, 1999). In a recent study, treatment of TNBS induced experimental colitis with poly (ADP-ribose) polymerase inhibitor resulted in reduced DNA-binding of both NF-κB and AP-1 in the colon (Zingarelli *et al.*, 2003). Although TFDG is known to inhibit the activation of both NF-κB and AP-1 (Chung *et al.*, 1999; Lin *et al.*, 1999), in the present study, we have focused on the mechanism of NF-κB activation in relation to the pharmacological effect of TFDG. In our previous study, we reported the protective effect of thearubigin in TNBS-induced colon injury in mice (Maity *et al.*, 2003) and suggested that inhibition of NF-κB-regulated transcriptional control of proinflammatory cytokines may be an important target for the treatment of IBD. Theaflavins and thearubigins are known to be the major polyphenols of black tea. They have phenol rings that act as electron traps to scavenge peroxy radicals, superoxide-anions and hydroxyl radicals (Katiyar *et al.*, 1993; Lin *et al.*, 1996). Among theaflavins, TFDG, which has two gallic acid moieties, exhibited the strongest anti-inflammatory activity as judged by its suppression on iNOS induction in LPS-stimulated macrophages. The inhibition of iNOS protein induction was in the following order: TFDG > (−) epigallocatechin-3-gallate > a mixture of theaflavin-3-gallate and theaflavin-3′-gallate > thearubigin > theaflavin (Lin *et al.*, 1999). Our results are also in agreement with this order.

The data presented in this report suggest that TFDG exerts its beneficial effect on TNBS-induced colitis mostly through its ability to inhibit the activation of NF-κB by inhibiting the phosphorylation and subsequent degradation of IκBα. The study is consistent with the findings that TFDG may exert its anti-inflammatory and cancer chemopreventive actions by suppressing the activation of NF-κB through inhibition of IKK activity (Pan *et al.*, 2000; Liang *et al.*, 2002). The degree of inflammation and tissue injury caused by TNBS was substantially reduced in mice treated with a dose of 5 mg kg^{-1} day^{-1} TFDG. This dose of TFDG may be correlated to an average consumption of four cups of tea per day for a 70-kg adult human as according to Lodovici *et al.* (2000).

There is ample evidence from animal models of IBD that NO is involved in gut inflammation through the modulation of iNOS (Rachmilewitz *et al.*, 1995; Singer *et al.*, 1996; Kimura *et al.*, 1997). Selective inhibition of iNOS can significantly decrease the extent and severity of tissue injury in experimental colitis (Kankuri *et al.*, 2001). The data presented in this paper confirm that oral administration of TFDG downregulates the TNBS-induced overexpression of proinflammatory cytokine genes for IFN-γ, IL-12, TNF-α and also iNOS. TFDG treatment led to a marked suppression in IL-12 mRNA levels by mucosal cells of TNBS-administered mice, which may result in reduced Th1 response as IL-12 production is critical for the development of Th1 cells (Trinchieri, 1998). A growing body of literature is available on the beneficial effect of EGCG, the most effective polyphenol component of green tea, on various disease processes and health disorders. Although limited studies have been carried out with black tea, most of the biological functions of tea polyphenols have been ascribed to their antioxidation activity and theaflavins in black tea possess at least the same antioxidant potency as catechins in green tea (Leung *et al.*, 2001). Thus, both EGCG and TFDG are known to inactivate PKC (Chen *et al.*, 1999)

and have also been shown to block the induction of NOS (Lin *et al*., 1999). Moreover, antitumor promotion effects of EGCG and TFDG may be functionally linked to the inhibition of signal transduction to transcription factors such as AP-1 and NF-κB (Nomura *et al*., 2000).

The mechanism by which TFDG inhibits iNOS, IL-12 and TNF-α production seems to be through the downregulation of NF-κB-mediated activation, since TFDG is capable of inhibiting NF-κB activity in EMSA using nuclear extracts of colonic cells. As NF-κB activation is believed to play a major role in regulation of proinflammatory gene transcription, its suppression by TFDG may inhibit early steps of inflammation and modulate upregulation of multiple proinflammatory genes. Binding sites for the NF-κB family of transcription factors are found in the promoter and enhancer regions of a multitude of genes, including cytokines, chemokines and growth factors that are known to be involved in the inflammatory response. Our data suggest that activation and nuclear translocation of NF-κB is important for TNBS-induced colon injury in mice. The accumulation of p65 and p50 in the nuclear fraction of TNBS-treated mice, were significantly decreased in TFDG-pretreated mice. Our findings also support a role for IKBα *in vivo* because activation of NF-κB during TNBS-induced colitis was accompanied by depletion of IκBα from the colonic tissue, presumably though proteolytic degradation. The data presented in this study thus strongly suggest that activation of NF-κB *in vivo* is a necessary prerequisite for the production of proinflammatory cytokines in TNBS-induced colitis and the protection afforded by TFDG may be due to its antioxidant effects. This is consistent with the observation of other *in vitro* and *in vivo* studies where treatments with antioxidants such as *N*-acetyl cysteine (Leff *et al*., 1993) and pyrrolidine dithiocarbamate (Nathens *et al*., 1997) were found to block activation of NF-κB by blocking the signal-induced phosphorylation of IκBα.

In conclusion, this study demonstrates that the degree of colitis caused by administration of TNBS is significantly attenuated by TFDG, a major polyphenol of black tea. Further, we have provided evidence that the activation of transcription factor NF-κB plays a significant role in TNBS-induced colitis and the therapeutic potential of TFDG resides in its ability to downregulate the activation of NF-κB by inhibiting the degradation of its endogenous inhibitor IκBα. TFDG thus may be used as a downregulator of NF-κB transactivation and might prove to be therapeutically effective for the treatment of inflammation in general.

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Conflict of interest

The authors state no conflict of interest.

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