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Thearubigin, the major polyphenol of black tea, ameliorates mucosal injury in trinitrobenzene sulfonic acid-induced colitis

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Abstract

Inflammatory bowel disease is characterized by oxidative and nitrosative stress, leukocyte infiltration and upregulation of proinflammatory cytokines. The aim of the present study was to examine the protective effects of thearubigin, an anti-inflammatory and anti-oxidant beverage derivative, on 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice, a model for inflammatory bowel disease. Intestinal lesions (judged by macroscopic and histological score) were associated with neutrophil infiltration (measured as increase in myeloperoxidase activity in the mucosa), increased serine protease activity (may be involved in the degradation of colonic tissue) and high levels of malondialdehyde (an indicator of lipid peroxidation). Both nitric oxide (NO) and O_2^- were increased with concomitant upregulation in the mRNA expression of proinflammatory cytokine response and inducible NO synthase (iNOS). Dose–response studies revealed that pretreatment of mice with thearubigin (40 mg kg^{-1} day^{-1}, i.e. for 10 days) significantly ameliorated the appearance of diarrhoea and the disruption of colonic architecture. Higher dose (100 mg kg^{-1}) had comparable effects. This was associated with a significant reduction in the degree of both neutrophil infiltration and lipid peroxidation in the inflamed colon as well as decreased serine protease activity. Thearubigin also reduced the levels of NO and O_2^- associated with the favourable expression of T-helper 1 cytokines and iNOS. Consistent with these observations, nuclear factor kappa B (NF-κB) activation in colonic mucosa was suppressed in thearubigin-treated mice. The results of this study suggest that thearubigin, the most predominant polyphenol of black tea, exerts beneficial effects in experimental colitis and may, therefore, be useful in the treatment of inflammatory bowel disease.

Keywords: Thearubigin; Inflammatory bowel disease; Black tea; Colitis; Cytokine; Nitric oxide (NO) synthase, inducible; NF-κB (nuclear factor kappa B)

1. Introduction

Inflammatory bowel disease, identified and diagnosed by a set of clinical, endoscopic, and histological features (Kirsner and Shorter, 1988), is of still unknown aetiology. Treating inflammatory bowel disease while limiting drug-induced toxicity is a continuous challenge. 5-Aminosalicyclic acid and salazosulphapyridine are the drugs of choice for current medical treatment. Corticosteroids, azathioprine, mercaptopurines and cyclosporine are used in more severe forms of the disease (Hanauer, 1996). Because of the lack of specific, curative treatments with limited toxicity, there is a pressing need for developing effective therapeutic approaches. Various models of inflammatory bowel disease have been found to be associated with an overproduction of nitric oxide (NO) due to the expression of the inducible isoform of NO synthase (iNOS) (Nathan, 1996). Increased luminal activities of NO have also been detected in ulcerative colitis (Lundberg et al., 1994) and in the colonic lavage fluid in different animal models of inflammatory bowel disease (Gunawardana et al., 1997; Feretti et al., 1997). Excessive production of NO in chronic colitis may be detrimental to the integrity of the colonic mucosa (McKenzie et al., 1996). In accordance with this information, NO-related treatments serve as a promising pharmacological approach in the treatment of these disease states (Rachmilewitz et al., 1995a; Neilly et al., 1995). Interventions, which reduce the generation or the

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effects of reactive nitrogen, exert beneficial effects in a variety of models of inflammation, including the trinitrobenzene sulfonic acid-induced colitis used here (Hogaboam et al., 1995; Miller et al., 1993; Neilly et al., 1996).

Alternative medicine is becoming an increasingly attractive approach for the treatment of various inflammatory disorders among patients unresponsive to or unwilling to take standard medicines. Among these alternative approaches is the use of natural products and dietary components, which have the advantage of being relatively nontoxic. However, limited scientific evidence regarding the effectiveness of these natural derivatives in conjunction with a lack of mechanistic understanding of their actions has prevented their incorporation into the mainstream of medical care. Over the last decade, there has been an increasing awareness of the potential health benefits of phytochemicals present in beverages, and in tea in particular. Tea is one of the most popular beverages of the world and about 80% of the dried tea manufactured annually is consumed as black tea (Katiyar and Mukhtar, 1996). Although polyphenols are the most significant group of tea components, the chemical compositions of green and black tea are different. The main phenolic components of green tea are catechins, in particular epicatechins, epicatechin gallate, epigallocatechin and epigallocatechin gallate. The production of black tea involves further processing, during which a substantial proportions of catechins are converted to theaflavins and thearubigins by a polyphenol oxidase (Balentine et al., 1997). While theaflavins consist of 1–2% of the total dry weight, about 10–20% of the dry weight of black tea is due to thearubigins, which are more extensively oxidized and polymerised, have a wide range of molecular weights, and are less well characterized. Recent advances in tea polyphenols have revealed diverse pharmacological effects including anti-inflammatory, antioxidative (Ho et al., 1992; Lin et al., 1996), antimutagenic (Shiraki et al., 1994) and anticarcinogenic (Yang and Wang, 1993) effects. A number of studies have shown that tea polyphenols including thearubigin, the major polyphenol of black tea, inhibit production of NO and expression of iNOS mRNA by murine macrophages (Kim et al., 1999; Chan et al., 1997; Lin et al., 1999).

Of the several animal models of intestinal inflammation, the well-characterized haptene reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis resembles human inflammatory bowel disease in terms of its various histological features including infiltration of colonic mucosa by neutrophils and macrophages and increased production of inflammatory mediators including T-helper 1 profile of cytokines (Parronchi et al., 1997). Moreover, various experimental trials using antibodies to interleukin-12, interleukin-4 gene transfer, and antisense oligonucleotides against nuclear factor kappa B (NF-κB) have indicated that the TNBS-induced colitis model is useful to test new therapeutic strategies for human (Fuss et al., 1999; Hogaboam et al., 1997; Neurath et al., 1996). Since black tea-derived thearubigins are a major source of dietary flavonoid having antioxidative and antinitrosative effects and since inflammatory bowel disease is associated with a marked upregulation of NO, it was thought worthwhile to investigate the effects of thearubigin on the inflammatory response (colitis) caused by intracolonic administration of TNBS.

2. Materials and methods

2.1. Animals and reagents

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, i.e. 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. Thearubigin was isolated from black tea according to the method described earlier (Chen and Ho, 1995). TNBS and all other chemicals were purchased from Sigma (USA).

2.2. Experimental colitis

Colitis was induced in mice by intrarectal administration of 0.1 ml of TNBS (60 mg ml^{-1} in 30% ethanol), through a trochar needle approximately 3–4 cm proximal to the anus according to the model described earlier (Neurath et al., 1995). Control mice received 30% ethanol in phosphate-buffered saline using the same technique. In the treated group of animals, thearubigin was given daily i.g. (10–100 mg kg^{-1} day^{-1}) for 10 days before subjecting the mice to TNBS-induced colitis and same dose of thearubigin was continued till the mice were sacrificed 8 days after the induction of colitis.

2.3. Macroscopic assessment of severity of colitis

Mice were sacrificed 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 or absence of ulcers. The criteria for assessing macroscopic damage was based on a semiquantitative 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 myeloperoxidase activity, malondialdehyde, NO and O_2^- production, and mRNA expression of cytokines and iNOS.

2.4. Microscopic assessment of colitis

The colon was fixed in 10% formalin in phosphate-buffered saline for 1 week and the samples were then dehydrated in graded ethanol and embedded in paraffin. Thereafter, 7-μm sections 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 the colon wall; and 4, transmural leukocyte infiltration, loss of goblet cells, high vascular density, and thickening of the colon wall.

2.5. Assessment of NO production

Tissues from the proximal third of the colon were homogenized in 40 mM HEPES containing 320 mM sucrose. Nitrite + nitrate production, an indicator of NO synthesis, was measured in the supernatant (10,000 × *g* for 20 min at 4 °C) according to Zingarelli et al. (1997). Nitrate in the supernatant was reduced to nitrite by incubation with nitrate reductase (670 mU ml^{-1}) and NADPH (160 mM) at room temperature for 3 h. One hundred microliters of the sample was then mixed with an equal volume of Griess reagent (1% sulphanilamide and 0.1% *N*-(1-napthyl)-ethylenediamine dihydrochloride in 5% H_3PO_4) and incubated at room temperature for 10 min. Absorbance at 540 nm was then measured. The amount of nitrite released was quantified by comparison with sodium nitrite as standard.

2.6. Estimation of O_2^-

Superoxide production was measured as described previously (Markert et al., 1984). Briefly, 10^6 extravasated neutrophils were incubated for 30 min at 37 °C in the presence of 2 μg ml^{-1} phorbol myristate acetate, 1 mg ml^{-1} cytochrome *c*, 30 μg ml^{-1} catalase, ± 100 μg ml^{-1} superoxide dismutase in D-glucose phosphate-buffered saline. The cells were then removed by centrifugation and the absorbance of reduced cytochrome *c* measured at 550 nm.

2.7. Myeloperoxidase activity

Myeloperoxidase is an enzyme found in cells of myeloid origin, and has been used extensively as a biochemical marker of granulocyte (mainly neutrophil) infiltration into gastrointestinal tissues (Morris et al., 1989). Samples of distal colon was homogenized in 10 mM potassium phosphate buffer, pH 7.0, containing 0.5% hexadecyl trimethyl ammonium bromide and centrifuged for 30 min at 20,000 × *g* at 4 °C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethyl benzidine and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. One unit of myeloperoxidase activity was defined as that degrading 1 μmol H_2O_2/min at 37 °C and was expressed as units per gram of tissue sampled (U g^{-1} tissue).

2.8. Malondialdehyde measurement

Malondialdehyde levels in the colon were determined as an indicator of lipid peroxidation (Ohkawa et al., 1979). The tissue was homogenized in 1.15% KCl solution. Homogenate (0.1 ml) was then added to a reaction mixture containing 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid, 1.5 ml of 0.8% thiobarbituric acid and 0.7 ml of distilled water. Samples were boiled for 1 h at 95 °C and centrifuged at 3000 × *g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

2.9. Analysis of protease activity

A small piece of colon tissue from the centre of the ulcer was homogenized for 10 s in cold phosphate-buffered saline. The homogenate was centrifuged (14,000 × *g* for 5 min at 4 °C) and the supernatant was analysed for protease activity on gelatin zymograms according to Hawkins et al. (1997). In short, 12% SDS-polyacrylamide gels were prepared containing 0.1% gelatin. An equal amount of protein from each sample (10–25 μg) was applied to the gel in standard SDS-gel loading buffer containing 0.1% SDS but lacking β-mercaptoethanol, and samples were not boiled prior to loading. After electrophoresis, gels were soaked in 2% Triton X-100 in distilled water with shaking for 15 min. Gels were then incubated in 50 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ for 12 h at 37 °C, stained in amido black and subsequently destained. Protease activity shows up as clear bands (indicative of cleavage of the gelatin substrate) on a blue background.

2.10. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of cytokine and iNOS mRNA

RT-PCR was performed in colon tissue samples from mice 4 days post-TNBS to determine the cytokine profile of mRNA for interferon-γ, interleukin-12 p40, interleukin-4, iNOS and β-actin. Reverse transcription of 1 μg of RNA was performed according to the manufacturer's protocol for the Superscript One-Step RT-PCR system (Life Technologies). The 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.

2.11. Electrophoretic mobility shift assay

The nuclear extracts were prepared from excised colon according to the method of Yang et al. (1998). For electro-

phoretic mobility shift assay, 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′-CGGG-GACTTTCCGCTGGGGACTTTCCGCTTGAGCT-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, 0.001 M EDTA).

2.12. Statistical analysis

Results are expressed as means ± 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 thearubigin-pretreated groups were compared against those for the TNBS-treated group with a two-sided Wilcoxon rank-sum test.

3. Results

3.1. Macroscopic and histological evaluation

Intracolonic administration of TNBS/ethanol resulted in extensive haemorrhagic and ulcerative damage to the distal colon as observed up to 8 days. Macroscopic examination of the distal colon and rectum from TNBS-treated mice revealed the presence of multiple mucosal erosions and ulcerations. The caecum, colon and rectum showed evidence of mucosal congestion, erosions and haemorrhagic ulcerations evaluated to a peak at 4 days as shown in the damage score (Fig. 1). The histological features included a transmural necrosis and oedema and a diffuse leukocyte cellular infiltrate in the submucosa (Fig. 2B). Control group gave rise to a very mild early damage of the colon. Treatment of mice with thearubigin (40 or 100 mg kg^{-1} day^{-1}) resulted in a significant decrease in the extent and severity of the injury of the large intestine as evidenced by macroscopic damage score (Fig. 1) as well as histopathological

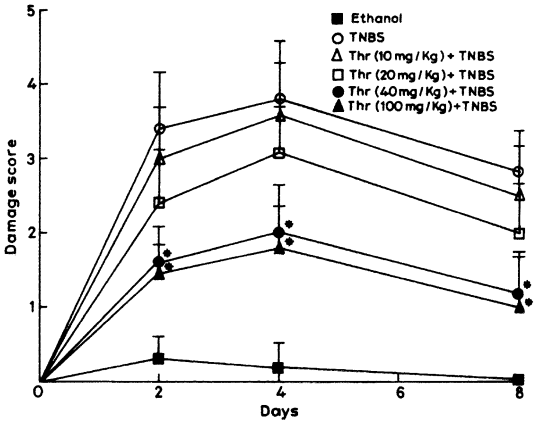

Fig. 1. Effect of thearubigin pretreatment on the macroscopic damage score of colonic tissues after induction of colitis. Mice were treated with 0.1 ml of TNBS (60 mg ml^{-1}) intracolonically and assessed at various times (2–8 days) after treatment. Colonic damage was scored on a scale of 0 (normal) to 5 (severe) by two independent observers. Values are means ± S.D. of 10 rats for each group. Results for the thearubigin-treated group were compared against those for the TNBS-treated group with a two-sided Wilcoxon rank-sum test. *$P < 0.001$ vs. TNBS.

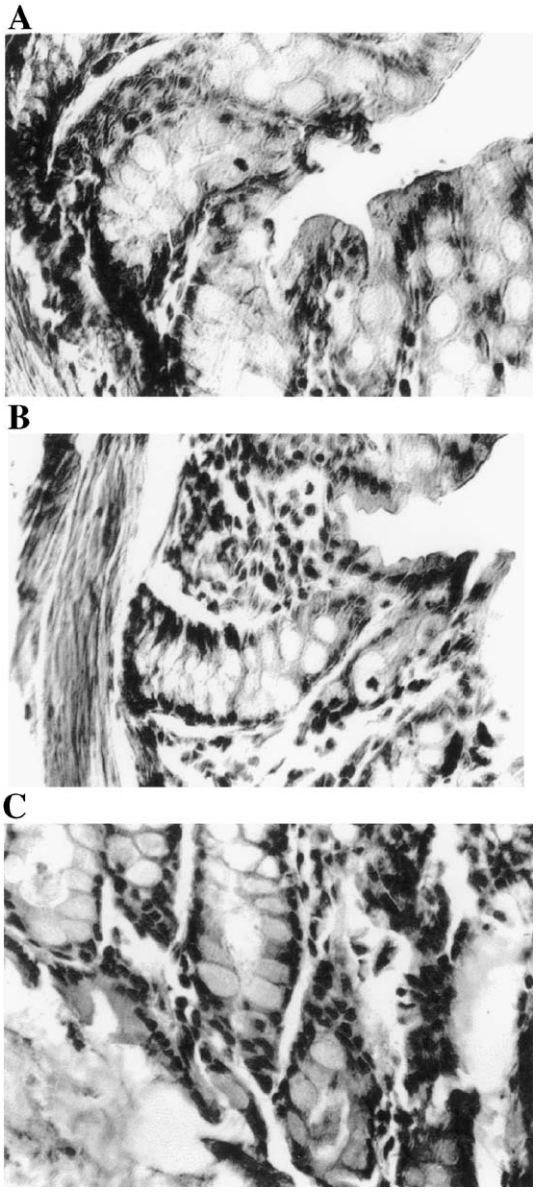

Fig. 2. Effect of thearubigin on colon injury. (A) Mucosal from control mice did not show any histological modifications. (B) TNBS-induced mucosal injury (at 4 days) associated with transmural necrosis and oedema and submucosal infiltration of inflammatory cells. (C) Pretreatment with thearubigin (40 mg kg^{-1}) prevented the disturbances in morphology associated with TNBS treatment. Original magnification: 500 ×.

assessment (Fig. 2C; Table 1). On the other hand, a dose of 10 or 20 mg kg^{-1} day^{-1} of thearubigin did not produce any significant effect on the severity of colitis compared to TNBS-treated group. The observed inflammatory changes of the large intestine were associated with an increase in weight of the colon and spleen as well as a significant decrease in body weight as compared to control mice (Fig. 3). In contrast, no significant increase of the weight was found in the colon and spleen of TNBS-treated mice, which has been pretreated with 40 or 100 mg kg^{-1} day^{-1} of thearubigin. Moreover, treatment with these dosages of thearubigin significantly reduced the loss in body weight, which correlated well with the amelioration of the colonic injury. However, the altered organ weight and body weight of TNBS-treated mice were not affected significantly by pretreatment of thearubigin with a dose of 10 or 20 mg kg^{-1} day^{-1} (Fig. 3). Thearubigin (40 or 100 mg kg^{-1}) pretreatment alone did not result in any change in the organ weight and body weight of control mice.

3.2. Generation of NO and O_2^-

Since infiltration of leukocytes into the mucosa has been suggested to contribute significantly to tissue necrosis and mucosal dysfunction of colitis by generating free radicals and oxidant molecules, both NO and O_2^- were measured. NO was measured in colonic biopsies whereas O_2^- was measured in extravasated neutrophils (Fig. 4). At 4 days after TNBS treatment, both NO and O_2^- levels were significantly elevated compared to control (7.3 ± 0.7 nmol mg^{-1} tissue and 4.5 ± 0.5 nmol min^{-1} 10^6 $cells^{-1}$ compared to 1.5 ± 0.3 nmol mg^{-1} and 1.1 ± 0.1 nmol min^{-1} 10^6 $cells^{-1}$, respectively; $P < 0.001$). Thearubigin pretreatment at dose levels of 40 and 100 mg kg^{-1} resulted in marked decrease of the elevated levels of both NO and O_2^- in the colon of TNBS-treated mice ($P < 0.01$). Pretreatment with 10 or 20 mg kg^{-1} of thearubigin though did not produce any significant effect on either NO or O_2^- production (Fig. 4). Moreover, thearubigin administration (40 and 100 mg kg^{-1}) in the absence of TNBS treatment as well as in 30% ethanol treatment showed comparable levels of NO and O_2^- as that of the control mice receiving 30% ethanol (data not shown).

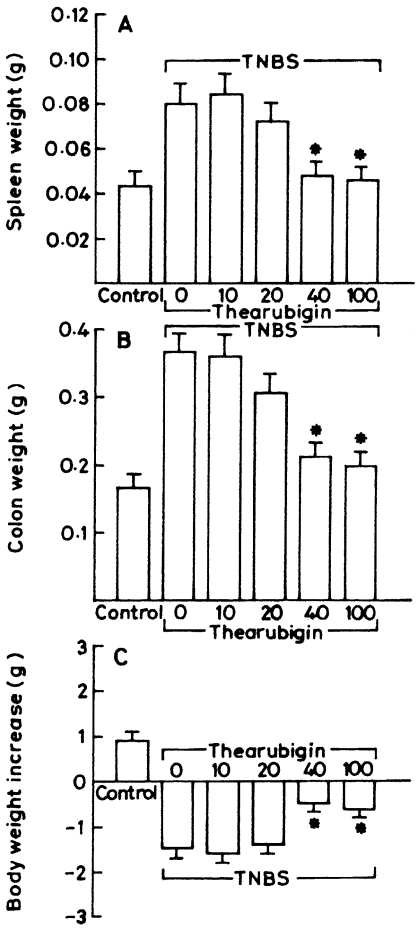

Fig. 3. Effect of thearubigin pretreatment on (A) spleen, (B) colon and (C) body weight. A significant increase in weight was observed at 4 days after TNBS administration in spleen and colon. Thearubigin pretreatment (40 and 100 mg kg^{-1}) significantly prevented the loss in body weight (C) as well as reduced the organ weight (A and B). Values are means ± S.D. of 10 rats for each group. *$P < 0.01$ vs. TNBS.

Table1
Effect of thearubigin pretreatment on colonic cytoprotection following administration of TNBS

	Colonic erosion							
	Scale					N	Mean	P^a
	0	1	2	3	4			
Control	6	4	0	0	0	10	0.40	
TNBS	0	1	2	4	3	10	2.90	
Thearubigin (10 mg kg^{-1})	0	2	4	2	2	10	2.60	0.6406
Thearubigin (20 mg kg^{-1})	0	7	2	1	0	10	2.20	0.1655
Thearubigin (40 mg kg^{-1})	0	7	3	0	0	10	1.40	0.0029
Thearubigin (100 mg kg^{-1})	0	6	3	1	0	10	1.30	0.0011

Histological scores graded from 0 to 4 as described in Materials and methods were carried out at 4 days after TNBS administration.

[a] Results for the thearubigin treatment group were compared against those for the TNBS group with a two-sided Wilcoxon rank-sum test. The histological scores for thearubigin (40 and 100 mg kg^{-1}) pretreated mice were significantly lower than that of untreated mice with TNBS-induced colitis ($P < 0.01$).

3.3. Effect of thearubigin on myeloperoxidase and malondialdehyde levels in TNBS-induced inflammatory bowel disease

Colonic injury by TNBS administration was also characterized by an increase in myeloperoxidase activity (28.0 ± 2.7 compared with 5.3 ± 1.4 U g^{-1} tissue in control), indicative of neutrophil infiltration in inflamed tissue (Fig. 5A) confirming the enhanced leukocyte infiltration seen at histological inspection. In this study, the extent of myeloperoxidase activity closely paralleled the increase of tissue malondialdehyde (34.7 ± 3.3 compared

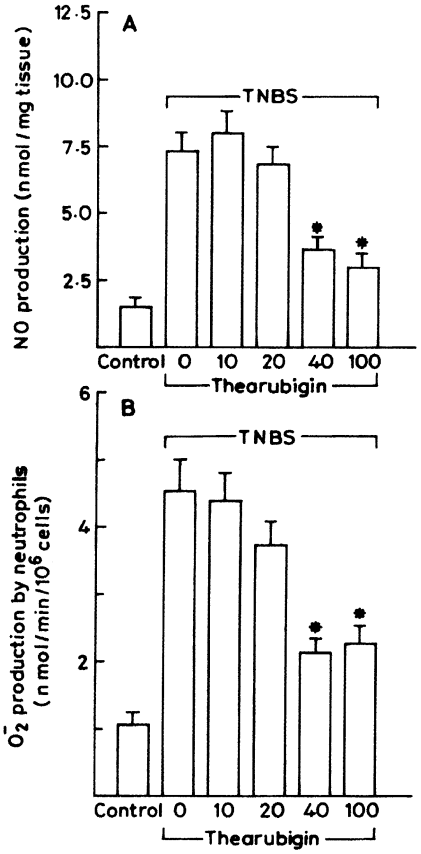

Fig. 4. Effect of thearubigin on NO and O_2^- production. (A) NO production in colonic tissue and (B) O_2^- production in neutrophils. TNBS treatment caused a significant increase of both NO and O_2^- generation, which were prevented by thearubigin pretreatment. Values are means ± S.D. of 10 rats for each group. *$P<0.01$ vs. TNBS.

with 7.3 ± 1.2 μM g^{-1} tissue in control), indicative of a massive lipid peroxidation (Fig. 5B). However, thearubigin pretreatment of TNBS-treated mice at dose levels of 40 and 100 mg kg^{-1} significantly prevented neutrophil infiltration, as assessed by myeloperoxidase activity ($P<0.01$) and also prevented the increased accumulation of malondialdehyde ($P<0.01$). Nevertheless, thearubigin (10 or 20 mg kg^{-1}) did not produce any significant change in the elevated levels of either myeloperoxidase or malondialdehyde when compared to TNBS-treated mice. Also, thearubigin (40 or 100 mg kg^{-1}) pretreatment in normal untreated mice as well as in 30% ethanol-treated mice showed comparable levels of myeloperoxidase and malondialdehyde as that of the control animals (data not shown).

3.4. Colonic serine protease activity

Since protease levels are known to be elevated in inflammatory bowel disease and thus may play a role in the extensive tissue damage in inflammatory bowel disease, protease activity in colon tissue was analysed. Colon tissue from control mice had little inherent protease activity (Fig. 6, lane 1), whereas this was markedly increased in TNBS-treated animals (lane 2). Significantly elevated levels of specific proteases of mass of ~ 112, 53 and 20 kDa were obtained from colonic mucosa of TNBS-induced colitis. Earlier studies on experimental model of inflammatory bowel disease using specific inhibitors of various proteases established that the majority of protease activity observed on the gelatin zymograms is due to serine proteases (Hawkins et al., 1997). However, thearubigin treatment (40 mg kg^{-1}) greatly reduced the degree of protease activity in the colon of mice, which had received TNBS to cause colitis (Fig. 6, lane 3). The suppression of serine protease activity in thearubigin-treated mice correlates well with the attenuation of mucosal injury in TNBS-induced colitis.

3.5. Cytokine production in treated mice

To gain an insight into the levels of various cytokines and iNOS after thearubigin treatment on TNBS-induced colitis, we examined the mRNA expression for a representative T-helper 1 cytokine (e.g. interferon-γ), a T-helper 1 inducer (e.g. interleukin-12), a T-helper 2 cytokine (interleukin-4) and iNOS, which catalyses the generation of NO from L-

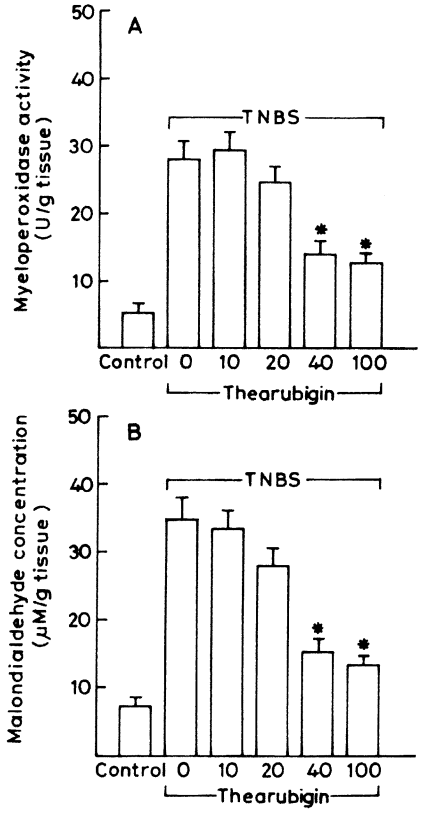

Fig. 5. Effect of thearubigin on neutrophil infiltration and lipid peroxidation. (A) Myeloperoxidase activity and (B) malondialdehyde levels in the colon. Myeloperoxidase activity and malondialdehyde levels were significantly increased in TNBS-treated mice in comparison to control. Thearubigin pretreatment showed a significant reduction of both myeloperoxidase activity and malondialdehyde levels. Values are means ± S.D. of 10 rats for each group. *$P<0.01$ vs. TNBS.

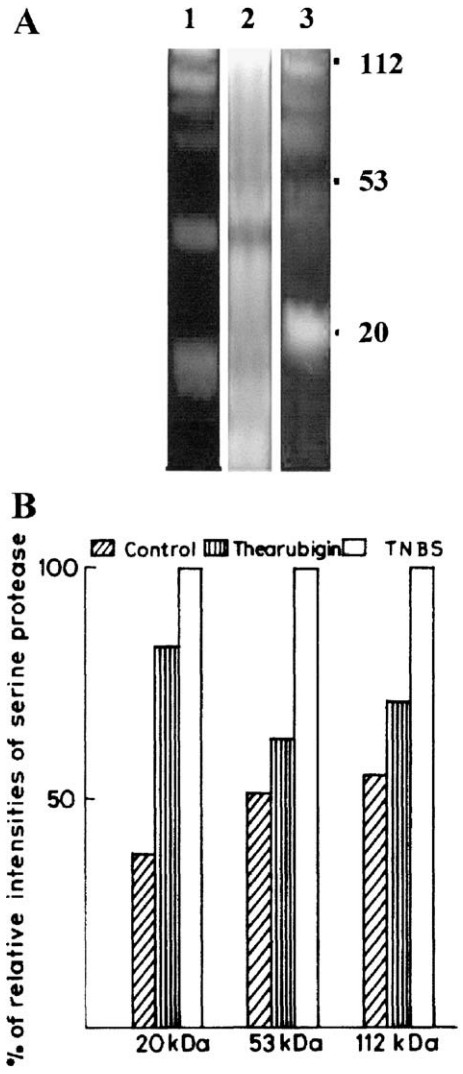

Fig. 6. Protease activity in TNBS-treated animals. (A) TNBS treatment showed a several-fold increase in protease activity (lane 2) over the untreated control animals (lane 1). Thearubigin-pretreated mice showed a marked reduction in protease activity (lane 3) compared to TNBS-treated mice. Numbers on the right indicate molecular mass in kDa. (B) Band intensities were quantified by densitometry.

arginine and plays a major role in inflammatory bowel disease. RT-PCR analysis of cytokine mRNA levels confirmed that experimental colitic mice treated with thearubigin could reverse an established T-helper 1 response into a possible T-helper 2 response (Fig. 7). Thus, mucosal cells from mice treated with TNBS contained significantly increased levels of interferon-γ and interleukin-12 p40 mRNAs than those from control representing a dominant inflammatory T-helper 1 response. However, thearubigin pretreatment resulted in marked suppression of both interferon-γ and interleukin-12 p40 mRNA levels with a little induction of interleukin-4 mRNA in TNBS-treated mice. In addition, the iNOS mRNA expression, which was very high in the mucosal cells of TNBS-treated mice, was significantly decreased by thearubigin pretreatment. These results suggest that inflammatory T-helper 1 functions have been effectively suppressed in BALB/c mice by thearubigin treatment so that T-helper 2 functions could possibly be activated to ameliorate mucosal injury in experimental colitis.

3.6. NF-κB in colonic mucosa of treated mice

To determine whether the decreased NO as well as iNOS mRNA in colonic mucosa was mediated through inhibition of iNOS transcription by suppression of NF-κB activation, we performed electrophoretic mobility shift assay using nuclear extracts of whole colonic tissues from control mice (which received 30% ethanol without TNBS) and thearubigin-treated or untreated TNBS colitis mice. The administration of TNBS alone enhanced NF-κB DNA binding activity of nuclear extracts in the inflamed colonic tissue, which was suppressed by pretreatment with either 40 or 100 mg kg^{-1} thearubigin. Excess unlabeled specific oligonu-

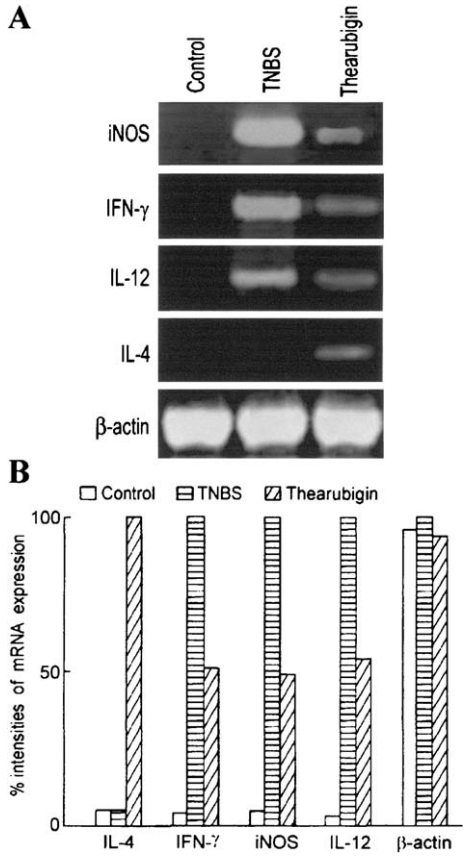

Fig. 7. Suppression of T-helper 1 phenotype in TNBS-treated mice subjected to thearubigin pretreatment (40 mg kg^{-1}) as analysed by RT-PCR. (A) Expression of interferon-γ, interleukin-12, interleukin-4, iNOS and β-actin mRNA by colon tissue samples of control and treated mice. RT-PCR products were visualized by ethidium bromide 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. (B) Band intensities were quantified by densitometry.

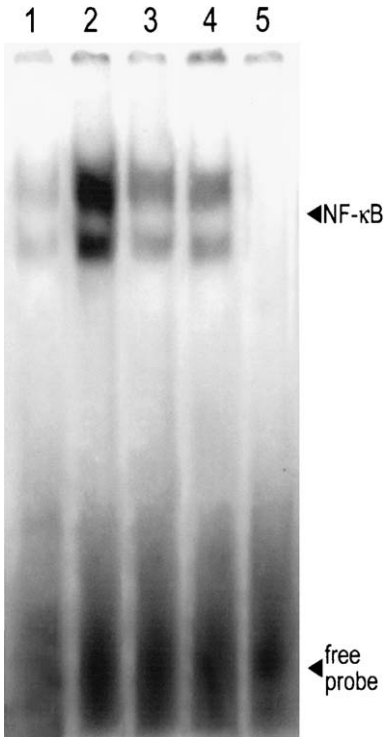

Fig. 8. Effect of thearubigin pretreatment on NF-κB activation. NF-κB activity was upregulated in nuclear extracts from TNBS-induced colonic mucosa and suppressed by thearubigin pretreatment. Nuclear extracts of the colonic tissue from control mice (lane 1); untreated mice with TNBS-induced colitis in the absence (lane 2) or presence (lane 5) of 50× molar excess of unlabeled probe; thearubigin (100 and 40 mg kg^{-1})-treated mice with TNBS-induced colitis (lanes 3 and 4) were analysed.

cleotides inhibited NF-κB mobility shift, thereby indicating the specificity of DNA–protein complex (Fig. 8).

4. Discussion

The use of natural anti-inflammatory products provides an attractive and safe alternative to modulate inflammatory disorders. The antioxidant activity of black and green tea, in terms of both quantity and quality, is higher than for almost all other commonly consumed beverages (Rice-Evans and Miller, 1996). Since polyphenols are known to be responsible for the antioxidative property of tea and since thearubigins are the most abundant polyphenols of black tea, it was thought worthwhile to study the effect of thearubigin on TNBS-induced experimental colitis, having close resemblance with human inflammatory bowel disease. The present study has demonstrated that TNBS causes a substantial degree of inflammation and tissue injury in the mouse colon, which is associated with an infiltration of the colon with polymorphonuclear cells (histology and myeloperoxidase activity) as well as lipid peroxidation. The degree of inflammation, tissue injury and lipid peroxidation caused by TNBS was substantially reduced in mice treated with a dose of 40 mg kg^{-1} day^{-1} of thearubigin. This dose of thearubigin 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). The impetus for this natural product therapy was the earlier observation that thearubigin could downregulate NO release from interferon-γ-activated macrophages (Sarkar and Bhaduri, 2001). Moreover, the therapy involving thearubigin pretreatment was effective in mice with ongoing disease in which a proinflammatory T-helper 1 response had been established. After treatment, the cytokine profile in these mice indicated a switch to suppression of proinflammatory T-helper 1 pattern.

Reactive NO radical is known to play a central role in human inflammatory bowel disease. Increased production of NO, and the presence of iNOS protein and iNOS mRNA have been demonstrated in affected areas of gut in patients suffering from ulcerative colitis or Crohn's disease (Singer et al., 1996; Rachmilewitz et al., 1995b; Kimura et al., 1997). On the other hand, based on data from lipopolysaccharide-induced inflammation, a concentration-dependent dual effect in the gut has been suggested (Laszlo and Whittle, 1995). Low production of NO by constitutive NOS may be protective and inhibitors of this physiological NOS have been reported to enhance intestinal lesions in inflammation (Laszlo and Whittle, 1995; Pfeiffer and Qiu, 1995). Prolonged production of high amounts of NO by iNOS on the other hand is proinflammatory and inhibition of iNOS seems to ameliorate the inflammatory response and tissue injury in experimental colitis (Hogaboam et al., 1995; McCafferty et al., 1997). An in vivo study by McKenzie et al. (1996) gives direct evidence on NO-induced injury on gut epithelial cells supporting the detrimental role of excessive NO in colitis. There is, therefore, good rationale to suggest that inhibition of excessive NO production by iNOS inhibitors will serve as promising approach in the management of inflammatory bowel disease. Enhanced NO generation as well as iNOS mRNA transcripts detected in the inflamed colonic segments may be attributed to the contribution of macrophages and inflammatory neutrophils since colonic NO generation has also been found to be stimulated by lipopolysaccharide and interferon-γ (Rachmilewitz et al., 1995a). We provide here the in vivo evidence that NO concentrations can be downregulated via suppression of proinflammatory cytokines by thearubigin in TNBS-induced colitis, resulting in a significant amelioration of the disease.

There is ample evidence in human inflammatory bowel disease that the inflammatory cytokines interleukin-1, tissue necrosis factors and interferon-γ are overexpressed, and this finding correlates with reports of excessive amounts of NO produced by activated iNOS in lamina propria mononuclear cells and colon epithelial cells (Singer et al., 1996; Godkin et al., 1996). This prompted us to investigate whether manipulation of cytokine profile by thearubigin would lead to reduced NO activities and thus decrease mucosal damage. Thearubigin treatment led to a marked suppression in the

interleukin-12 mRNA transcript by mucosal cells of TNBS-administered mice, resulting in a reduced ability to induce interferon-γ and an increased ability to induce interleukin-4 in CD4+ T cells. These results suggest that thearubigin-mediated inhibition of interleukin-12 production led to the inhibition of T-helper 1 and a possible enhancement of T-helper 2 cytokine synthesis in CD4+T cells. Since the cytokine profile of T-helper cells plays an important role to determine the outcome of many diseases, thearubigin may have therapeutic potential to treat T-helper 1-mediated diseases such as type-1 diabetes, multiple sclerosis, rheumatoid arthritis and inflammatory bowel disease. The mechanism by which thearubigin inhibits interleukin-12 production seems to be through the downregulation of NF-κB-mediated activation and binding to the p40-κB site, since thearubigin is capable of inhibiting NF-κB activity in electrophoretic mobility shift assay using nuclear extracts of whole cells of the colonic tissue. Theaflavin, another black tea polyphenol, has recently been shown to inhibit the activation of NF-κB (Lin et al., 1999). Since NF-κB activation is believed to play a major role in the regulation of proinflammatory gene transcription, therefore, by suppressing it thearubigin may inhibit early steps of inflammation and modulate upregulation of multiple proinflammatory genes.

It may be mentioned that scavengers of reactive oxygen including hydrogen peroxide, superoxide anions and hydroxyl radicals also reduce the tissue injury associated with inflammatory bowel disease suggesting that, in addition to reactive nitrogen, reactive oxygen species also play important role in the pathophysiology associated with this model of inflammation (Cuzzocrea et al., 2000). In addition to reactive oxygen, peroxynitrite ($ONOO^-$) is also generated in inflammatory bowel disease (Zingarelli et al., 1998). Reactive oxygen and $ONOO^-$ produce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage. The curative effect of thearubigin accompanied by reduced levels of superoxide anions, nitric oxide, malondialdehyde and serine protease is suggestive of the scavenging capability of both reactive nitrogen and oxygen species by this beverage product.

Assessment of the physiological relevance of the findings reported here must take into account the concentrations at which inhibitory or stimulatory effect of tea-derived polyphenols on cytokine production was observed. In a recent study, the effects of four major green tea-derived catechins and a black tea extract on the production of pro- and anti-inflammatory cytokines by human leukocytes in vitro were investigated (Crouvezier et al., 2001). Although the cytokine profile suggested anti-inflammatory properties of the tea-derived catechins, they were observed at concentrations that were unlikely to be achievable in plasma in vivo and are therefore unlikely to contribute to the protective effects of tea-derived polyphenols in inflammatory diseases. However, it is important to realize that due to inadequate bioavailability studies and in vitro experiments in isolation, very little is known about concentrations of these compounds in the small intestine and colon. It is possible that concentrations of catechins at these sites are much higher than in plasma and in this light, the effects of tea-derived polyphenols on the inflammatory response may yet be relevant in inflammatory disease of the bowel.

In conclusion, this study demonstrates that the degree of colitis caused by administration of TNBS is significantly attenuated by the major tea polyphenol radical scavenger thearubigin. The anti-inflammatory effects of thearubigin are associated with a reduction in (i) upregulation of proinflammatory T-helper 1 cytokine response leading to the suppression of iNOS and attenuation of the recruitment of neutrophils, (ii) lipid peroxidation and (iii) ultimately tissue injury. The results suggest that thearubigin may be useful in inflammatory bowel disease and other conditions associated with systemic inflammation.

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