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Protective role of cyclooxygenase inhibitors in the adverse action of passive cigarette smoking on the initiation of experimental colitis in rats

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Abstract

Clinical and experimental findings had indicated that cigarette smoke exposure, and cyclooxygenase-2, are strongly associated with inflammatory bowel disease. The present study aimed to evaluate the role of cyclooxygenase-2 in the pathogenesis of experimental inflammatory bowel disease as well as in the adverse action of cigarette-smoke exposure. Rats were pretreated with different cyclooxygenase-2 inhibitors (indomethacin, nimesulide, or SC-236 (4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide)) along with cigarette-smoke exposure before 2,4,6-trinitrobenzenesulfonic acid-enema. Results indicated that pretreatment with cyclooxygenase-2 inhibitors not only protected against 2,4,6-trinitrobenzenesulfonic acid-induced inflammatory bowel disease, but also attenuated the potentiating effect of cigarette-smoke exposure on colonic damage. Furthermore, the colonic cyclooxygenase-2 protein and mRNA expression was markedly induced by 2,4,6-trinitrobenzenesulfonic acid-enema, and it was potentiated further by cigarette-smoke exposure, while the cyclooxygenase-1 expression was not changed. The present study suggests that the highly induced cyclooxygenase-2 expression not only plays a pathogenic role in 2,4,6-trinitrobenzenesulfonic acid-induced inflammatory bowel disease, but also contributes to the adverse action of cigarette-smoke exposure on this disorder.

Keywords: Cigarette smoke; Inflammatory bowel disease; Cyclooxygenase-2; 2,4,6-Trinitrobenzene sulfonic acid; Cyclooxygenase inhibitor

1. Introduction

Inflammatory bowel diseases, which include ulcerative colitis and Crohn's disease, are chronic nonspecific inflammatory disorders with unknown cause. It had been found that cyclooxygenase-2 protein and mRNA expression were induced in inflammatory area of the colonic tissue in active inflammatory bowel disease patients, while cyclooxygenase-1 expression was not changed under colitis condition (Hendel and Nielsen, 1997; Singer et al., 1998). Moreover, it was also demonstrated that there is a clear relationship between endoscopic activity of the colitis and the relative presence of mRNA for cyclooxygenase-2, indicating that cyclooxygenase-2 was involved in the acute inflammatory response of chronic inflammatory bowel disease (Hendel and Nielsen, 1997). Cyclooxygenase is the key enzyme in the synthesis of prostanoids, such as prostaglandin E_2, prostaglandin I_2 and thromboxane A_2 from arachidonic acids in cell membrane. There are two isoforms of cyclooxygenase: cyclooxygenase-1 is a constitutive enzyme expressed in many tissues including the intestine and colon, whereas cyclooxygenase-2 is an inducible enzyme activated by many inflammatory stimuli, such as pro-inflammatory cytokines and some growth factors, and expressed in macrophages, fibroblasts, and other cell types during inflammation (Crofford, 1997; Rzymkiewicz et al., 1994). The induction of colonic cyclooxygenase-2 expression in ulcerative colitis and Crohn's disease patients may explain the increased levels of prostaglandin E_2 in the inflamed mucosa (Sharon and Stenson, 1984). In addition, some drugs currently used for the treatment of inflammatory bowel disease have been shown to have beneficial effects in experimental inflammatory bowel disease, ac-

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companied by the reduction of prostaglandin E_2, prostaglandin I_2 and thromboxane A_2 synthesis (Hillier et al., 1991; Zijlstra et al., 1993).

However, it is still controversial up to now whether cyclooxygenase-2 plays a crucial role in the pathogenesis of inflammatory bowel disease. An in vitro study clearly demonstrated that pretreatment with indomethacin, which is a nonspecific cyclooxygenase inhibitor, significantly prevented the acute injury of human colonic cells stimulated by incubation with 2,4,6-trinitrobenzene sulfonic acid (Stratton et al., 1996). This observation indicates that an acute injury of colonic cells produced by 2,4,6-trinitrobenzene sulfonic acid is likely to be mediated by the cyclooxygenase enzyme. On the contrary, it was found that treatment with indomethacin or some other selective cyclooxygenase-2 inhibitors twice daily after the induction of experimental inflammatory bowel disease by 2,4,6-trinitrobenzene sulfonic acid-enema resulted in an exacerbation of inflammation-associated colonic injury in rats (Wallace et al., 1992; Reuter et al., 1996), indicating that cyclooxygenase-2 could contribute in part to the recovery of inflammatory bowel disease. These controversial findings suggested that cyclooxygenase-2 might possibly play differential roles in the pathogenesis and healing process of inflammatory bowel disease. However, there is no direct evidence to consolidate the role of cyclooxygenase-2 in the initiation of inflammatory bowel disease.

Interestingly, it has been found that cigarette smoking (including passive cigarette smoking) is strongly associated with the pathogenesis of inflammatory bowel disease, which has much stronger adverse association with Crohn's disease in humans (Franceschi et al., 1987; Cosnes et al., 1996; Lashner et al., 1993). Consistently, our previous study has demonstrated that cigarette-smoke exposure could indeed potentiate the pathogenesis of experimental inflammatory bowel disease induced by 2,4,6-trinitrobenzene sulfonic acid–ethanol enema in rats (Guo et al., 1999). However, there is no report related to the connection between cyclooxygenase-2 expression and the adverse action of cigarette-smoke exposure on experimental inflammatory bowel disease.

Thus, we made use of this model not only to investigate whether cyclooxygenase-2 expression was indeed responsible in the initiation of 2,4,6-trinitrobenzene sulfonic acid-induced inflammatory bowel disease, but also clarify further whether this enzyme was also involved in the adverse action of cigarette-smoke exposure on this disorder in rats. We pretreated rats with nonselective cyclooxygenase-2 inhibitor indomethacin, moderately cyclooxygenase-2 selective inhibitor nimesulide as well as a newly synthesized cyclooxygenase-2 specific inhibitor SC-236 (4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide) together with cigarette-smoke exposure, in addition to measure the cyclooxygenase-2 protein and mRNA expression during the initiating processes of the experimental inflammatory bowel disease.

2. Materials and methods

2.1. Animals and reagents

The Committee on the Use of Live Animals had approved the present study in the University of Hong Kong. Male Sprague–Dawley rats (180–200 g) were fed a standard laboratory diet (Ralston Purina, Chicago, IL, USA), and given tap water (ad libitum). They were kept in a room under controlled temperature (22 ± 1°C), humidity (65–70%), and day/night cycle (12/12-h light/dark). Indomethacin was dissolved in absolute ethanol with dilution of Tween 80 (5%, v/v). Nimesulide was suspended in normal saline with several drops of Tween 80. SC-236, which was kindly provided by Searle (Chicago, IL, USA), was dissolved in absolute ethanol with dilution of Tween 80 (5%, v/v). 2,4,6-Trinitrobenzene sulfonic acid, indomethacin, nimesulide and all other chemicals used in this study were purchased from Sigma (St. Louis, MO, USA), unless specified otherwise.

2.2. Induction of experimental inflammatory bowel disease and assessment of colonic damage in rats

According to the well-established inflammatory bowel disease model described by Morris et al. (1989), rats were lightly anesthetized by diethyl ether (BDH, Poole, UK). Two hundred and fifty microliters of 50% (v/v) ethanol (E. Merck Darmstadt, Germany) containing 30 mg 2,4,6-trinitrobenzene sulfonic acid was slowly injected into the lumen of the colon, 8 cm proximal to the anus through the rectum using a polyethylene catheter (10-cm long, 2-mm external diameter) fitted onto a 1-ml syringe. Sham-operated animals in the normal control group received the same procedure but were administered with normal saline instead.

After being weighed, the rats were killed 24 h afterwards by deep diethyl ether anesthesia, following whole blood collection from the abdominal aorta. The distal colon was removed, opened and rinsed thoroughly in ice-cold normal saline. The lesion area was recorded and then measured using a 1-mm^2 grid by an observer who was unaware of the treatment. The distal colon with the length of 8 cm was weighed. The ratio of colon weight/body weight was used as a parameter to assess the degree of colon edema, which reflected the severity of colonic inflammation. One piece of colonic tissue (3×10 mm) from the most severe part of the colon was excised and fixed in 10% formalin buffer for subsequent immunohistochemical studies. The rest of the colonic tissue was collected, immediately frozen in liquid nitrogen and stored at -70°C until assayed for biochemical parameters. Colonic myeloperoxidase activity was determined within a week.

2.3. Cigarette-smoke exposure

The method for cigarette-smoke exposure was described in details previously (Chow et al., 1996; Guo et al., 1999). Commercial cigarettes (Camel, nonfilter, R.J. Reynolds, Winston-Salem, NC, USA) were used throughout the experiment. The rats in smoking group were exposed to freshly prepared cigarette smoke (4%, v/v: smoke/air) for 1-h period each time once daily for four consecutive days before the experimental inflammatory bowel disease induction. Control rats were subjected to the same procedures, but breathing only fresh air.

2.4. Drug administration

In the first part of the experiment, rats were pretreated with a nonselective cyclooxygenase-2 inhibitor indomethacin subcutaneously at dose of 2.5 mg/kg (Mann and Demers, 1983). Rats were divided into four groups of six to eight rats per group, including the control group (2,4,6-trinitrobenzene sulfonic acid + vehicle), Indo group (2,4,6-trinitrobenzene sulfonic acid + indomethacin), passive smoking with TNBS-ethanol group (smoke + 2,4,6-trinitrobenzene sulfonic acid + vehicle) and passive smoking with Indo group (smoke + 2,4,6-trinitrobenzene sulfonic acid + indomethacin). In the passive smoking groups, rats were exposed to cigarette smoke (4%, v/v: smoke/air) in a 1-h period once daily over four consecutive days before 2,4,6-trinitrobenzene sulfonic acid–ethanol enema. 2,4,6-Trinitrobenzene sulfonic acid–ethanol was administrated to the rats as described above 1 h after the last cigarette-smoke exposure. Indomethacin (2.5 mg/kg, s.c.) was administered to rats once daily for 4 days just before each cigarette-smoke exposure. In the second part of the experiment, in order to find out whether cyclooxygenase-2 is involved, rats were pretreated with nimesulide subcutaneously (25 mg/kg, s.c.) (Okajima et al., 1998; Hirata et al., 1997), which is a moderately selective inhibitor of cyclooxygenase-2 over cyclooxygenase-1 (about five times), under the same protocol as for indomethacin. In the third part of the experiment, after being demonstrated that cyclooxygenase-2 was only highly expressed after 2,4,6-trinitrobenzene sulfonic acid-enema, SC-236 (2.5 mg/kg, i.p.), a new specific inhibitor of cyclooxygenase-2 (Gierse et al., 1996; Penning et al., 1997), being approximately 1780 times more active on cyclooxygenase-2 than cyclooxygenase-1, was used. It was administered intraperitoneally 1 h before and 2 h after 2,4,6-trinitrobenzene sulfonic acid-enema. The doses of the three drugs are equipotent based on their IC_{50} value of cyclooxygenase-2 inhibition in vitro (Pairet et al., 1998; Gierse et al., 1996; Penning et al., 1997). Rats were all killed 24 h after 2,4,6-trinitrobenzene sulfonic acid–ethanol enema to evaluate the drug effects on the formation of colonic lesion and inflammation.

2.5. Measurement of myeloperoxidase activity in the colonic tissue

Myeloperoxidase activity was determined by a modified method described by Suzuki et al. (1983). In brief, colonic tissue was minced with scissors and homogenized with a homogenizer (Ultra-Turrax, Janke & Kunkel, Staufen, Germany) for 40 s in an ice-cold 50 mM phosphate buffered saline (pH 6.0) solution containing 0.5% hexadecyltrimethylammonium bromide. The homogenate was freeze–thawed three times, followed by repeated sonication for 30 s each. The detailed procedures of myeloperoxidase activity assay have been described previously (Guo et al., 1999). The final value was represented as U/g tissue.

2.6. Measurement of leukotriene B_4 and prostaglandin E_2 concentration in the colonic tissue

The colon tissue sample (100–150 mg) was scissored and homogenized in a Tris–HCl buffer solution (50 mM, pH 7.4) containing 100 mM NaCl, 1 mM $CaCl_2$, 1 mg/ml D-glucose and 28 μM indomethacin for 40 s. The homogenate was centrifuged (Beckman J2-21 centrifuge, Beckman Instrument, Fullerton, CA, USA) at 10,000 rpm for 15 min at 4°C. The supernatant was assayed using a leukotriene B_4 or prostaglandin E_2 immunoassay kit (R&D Systems, Minneapolis, MN, USA), followed by measuring with a microplate reader (Dynex Technologies, The Microtiter, Chantilly, Virginia, USA) at 405 nm. The protein content assay was based on the method of Dye-reagent (Read and Northcote, 1981) using a standard protein (Bovine albumin, 10 g/l, Sigma) as standard. The final value for samples was expressed as pg/mg protein.

2.7. Determination of prostanoids synthesis ex vivo from colonic tissue

After the colonic lesion was recorded, the colonic tissue sample (200–250 mg) was weighed, placed in an Eppendorf tube, and finely minced with scissors for 15 s. The sample was then suspended in 1.0 ml of Tris–HCl buffer (0.05 M; pH 7.4), incubated in a shaking water bath for 20 min (37°C), followed by centrifugation for 60 s in a centrifuge (Beckman GS-15R centrifuge, Germany, 9000 $\times g$, 4°C) (Wallace et al., 1992). The supernatant was then frozen (−20°C for no more than 1 week) for subsequent prostaglandin E_2, 6-keto-prostaglandin $F_{1\alpha}$ (a stable product of prostacyclin) and thromboxane B_2 (a stable product of thromboxane A_2) immunoassay (Immunoassay kits, R&D System). The final value was expressed as pg/mg wet tissue.

2.8. Immunohistochemistry detection of cyclooxygenase-2 in colonic tissue

Fixed tissue sections (5 μm) were mounted on Vectabond Reagent-coated slides, deparaffinized and rehy-

drated through xylene, graded ethanol to distilled water. After blocking endogenous peroxidase with 0.3% hydrogen peroxide in methanol for 40 min, sections were digested with 0.1% trypsin for 15 min. After nonspecific binding with 0.05 M phosphate-buffered saline solution containing 3% normal horse serum and 0.3% Triton X-100 for 30 min, sections were rinsed with PBS, incubated with goat anti-rat cyclooxygenase-2 polyclonal antibody (M-19) (Santa Cruz Biotechnology, USA, cat # sc-1747) (at dilution of 1:400) overnight at 4°C in a humidity chamber. The secondary, biotinylated antibody (LSAB kit, DAKO, Denmark) was then applied for 30 min followed by rinsing with phosphate-buffered saline. Staining was performed by the addition of streptavidin (LSAB kit, DAKO, Denmark) for 30 min, rinsed in phosphate-buffered saline and developed in 3,3′-diaminobendine tetrahydrochloride for less than 3 min. Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted.

2.9. Detection of cyclooxygenase protein expression by Western blotting

The colonic tissues were homogenized at 4°C in RIPA buffer solution (50 mmol/l Tris–HCL, pH 7.5, 150 mmol/l NaCl, 0.1% sodium dodecyl sulfate, 0.5% acholate, 2 mmol/l EDTA, 1% Triton X-100, 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin. After centrifugation at $10{,}000 \times g$ at 4°C for 20 min, the supernatant (100 μg of total protein) was denatured and separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was probed with a goat polyclonal antibody against rat cyclooxygenase-1 or cyclooxygenase-2 (cyclooxygenase-1, M-20, Cat # sc-1754; cyclooxygenase-2, M-19, Cat. # sc-1747, Santa Cruz Biotechnology), developed by the Enzyme chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, USA) and exposed to X-ray film. Protein determinations were made with Bio-Rad protein assay kit with bovine serum albumin as a standard. Prestained molecular-weight standards (Bio-Rad) were used as markers. Quantification was carried out by video densitometry.

2.10. Detection of cyclooxygenase mRNA expression by reverse transcriptase-polyermerase chain reaction (RT-PCR)

Total RNA was isolated from the colonic tissue of rats by using Trizol reagent (Gibco BRL, Gaithersburg, MD, USA). First-strand complementary DNAs were synthesized from 5 μg RNA by using oligo dT primer and thermoscript reverse transcriptase-polymerase chain reaction (RT-PCR) system (Gibco BRL, Gaithersburg, MD, USA). The PCR cycle was performed for cyclooxygenase-1, cyclooxygenase-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the same complementary DNA sample by using a PCR Thermal Cycler (Gene Amp PCR System 9700, The Perkin-Elmer, Norwalk, CT, USA). The sequence of the oligonucleotide primers are as follows: sense cyclooxygenase-1, 5′ CTG CTG AGA AGG GAG TTC CAT 3′; antisense cyclooxygenase-1, 5′ GTC ACA CAC ACG GTT ATG CT 3′; sense cyclooxygenase-2, 5′ ACA CTC TAT CAC TGG CAT CC 3′, and antisense cyclooxygenase-2, 5′ GAA GGG ACA CCC TTT CAC AT 3′, as described by Yang et al. (1998). Concurrent RT-PCR amplification of GAPDH as a housekeeping gene was used to control for variations in the efficiencies of RNA isolation and RT. PCR cycle was an initial step of 94°C for 3.5 min, followed by 94°C for 1.5 min, 54°C for 1.5 min, 72°C for 1.5 min of 30 cycles for COX-2 and GAPDH, 45 cycles for cyclooxygenase-1 and a final cycle of 72°C for 10 min. The PCR products—a 398-base pairs cyclooxygenase-1 fragment, a 584-base pairs cyclooxygenase-2 fragment and a 309-base pairs GAPDH fragment were then visualized by ultraviolet illumination after electrophoresis through 1% agarose gels containing 0.5 μg/ml

Table 1
Effect of pretreatment with indomethacin on experimental inflammatory bowel disease in normal or passive cigarette smoking rats

Group	Body weight change (g)	Colon weight/body weight ($\times 10^{-2}$)	Lesion area (mm^2)	Myeloperoxidase activity (U/g tissue)
Control + Vehicle	-24.0 ± 1.7	0.81 ± 0.03	393.50 ± 49.97	12.42 ± 2.22
Control + Indo	-5.0 ± 1.4[a]	0.59 ± 0.02[a]	351.75 ± 25.36	3.60 ± 1.02[b]
Smoke + Vehicle	-22.5 ± 2.7	0.94 ± 0.03[b]	557.88 ± 37.51[c]	22.07 ± 3.58[c]
Smoke + Indo	-1.3 ± 0.8[d]	0.59 ± 0.03[d]	244.12 ± 25.22[d]	2.24 ± 0.33[d]

Indomethacin (Indo, 2.5 mg/kg, s.c.) or 5% (v/v) of Tween 80 with 1% (v/v) of ethanol (Vehicle, 2 ml/kg, s.c.) was pre-administered subcutaneously in normal (Control) or passive cigarette smoking (Smoke) rats once daily for 4 days each time before cigarette smoke (4%, v/v, smoke/air) exposure. Rats were killed 24 h after 2,4,6-trinitrobenzene sulfonic acid/ethanol enema to assess the colonic lesion and inflammation. Each value is expressed as mean ± S.E.M., $N = 6–8$/group.

[a] $P < 0.001$, compared with the value of corresponding control vehicle group.

[b] $P < 0.01$, compared with the value of corresponding control vehicle group.

[c] $P < 0.05$, compared with the value of corresponding control vehicle group.

[d] $P < 0.001$, compared with the value of corresponding smoke vehicle group.

Table 2
Effect of pretreatment with nimesulide on experimental inflammatory bowel disease in normal or passive cigarette smoking rats

Group	Body weight change (g)	Colon weight/body weight ($\times 10^{-2}$)	Lesion area (mm^2)	Myeloperoxidase activity (U/g tissue)
Control + Vehicle	-17.3 ± 3.9	0.62 ± 0.05	293.67 ± 56.65	7.60 ± 1.47
Control + Nime	-6.0 ± 1.4[a]	0.45 ± 0.03[b]	187.00 ± 47.34[a]	3.45 ± 0.53[a]
Smoke + Vehicle	-20.5 ± 2.7	0.76 ± 0.03[a]	447.11 ± 51.21[a]	11.89 ± 2.26[a]
Smoke + Nime	-8.5 ± 2.4[c]	0.53 ± 0.06[c]	411.00 ± 77.78	5.15 ± 1.47[c]

Nimesulide (Nime, 25 mg/kg, s.c.) or normal saline with 1% (v/v) of Tween 80 (Vehicle, 2 ml/kg, s.c.) was pre-administered subcutaneously in normal (Control) or passive cigarette smoking (Smoke) rats once daily for 4 days each time before cigarette smoke (4%, v/v, smoke/air) exposure. Rats were killed 24 h after 2,4,6-trinitrobenzene sulfonic acid/ethanol enema to assess the colonic lesion and inflammation. Each value is expressed as mean ± S.E.M., $N = 6–8$/group.

[a] $P < 0.05$, compared with the value of corresponding control vehicle group.
[b] $P < 0.01$, compared with the value of corresponding control vehicle group.
[c] $P < 0.05$, compared with the value of corresponding smoke vehicle group.

ethidium bromide. The gel photographs were scanned with a computerized densitometer.

2.11. Statistical analysis

Results were expressed as means ± standard error of the mean (S.E.M.). Differences between two groups were examined using one-way analysis of variance (ANOVA) followed by unpaired Student's *t*-test unless specified. A probability (P value) of less than 0.05 was considered significant.

3. Results

3.1. Effects of pretreatment with cyclooxygenase-2 inhibitors on the colonic lesion and inflammation and myeloperoxidase activity in 2,4,6-trinitrobenzene sulfonic acid-induced inflammatory bowel disease together with cigarette-smoke pre-exposure

2,4,6-Trinitrobenzene sulfonic acid-enema resulted in severe colonic lesion and inflammation as indicated by body weight loss, increase of the ratio of colon weight/body weight (index of colonic edema), lesion area and myeloperoxidase activity (a quantitative index of inflammation and a marker of neutrophil infiltration) (Tables 1–3). Pre-exposure of cigarette smoke (4%, v/v) significantly increased the lesion area, ratio of colon weight/body weight as well as myeloperoxidase activity in the colonic tissues as compared with control group (Tables 1–3). Pretreatment with indomethacin (2.5 mg/kg, s.c.) significantly alleviated the body weight loss, and decreased the ratio of colon weight/body weight and myeloperoxidase activity in colonic tissue in the 2,4,6-trinitrobenzene sulfonic acid-enema treated animals. Moreover, in the passive smoking group, indomethacin produced even much more protective effect on 2,4,6-trinitrobenzene sulfonic acid-induced colonic lesion and inflammation in addition to marked attenuation of the aggravating action of cigarette-smoke exposure on the colonic damage (Table 1). The potentiation of colonic myeloperoxidase activity by cigarette-smoke exposure was also dramatically attenuated by indomethacin pretreatment (Table 1). At the mean time, the colonic prostaglandin E_2 concentration was reduced to

Table 3
Effect of administration of SC-236 on experimental inflammatory bowel disease in normal or passive cigarette smoking rats

Group	Body weight change (g)	Colon weight/body weight ($\times 10^{-2}$)	Lesion area (mm^2)	Myeloperoxidase activity (U/g tissue)
Control + Vehicle	-21.2 ± 1.2	0.87 ± 0.06	404.4 ± 38.35	8.60 ± 0.48
Control + SC-236	-1.6 ± 1.9[a]	0.61 ± 0.03[a]	306.00 ± 42.70	4.43 ± 1.43[b]
Smoke + Vehicle	-21.0 ± 0.86	0.95 ± 0.02	548.00 ± 46.67[b]	10.89 ± 0.57[b]
Smoke + SC-236	-7.0 ± 2.1[c]	0.65 ± 0.05[c]	356.50 ± 65.36[d]	4.51 ± 1.08[e]

SC-236 (2.5 mg/kg, i.p.) or normal saline containing with 5% (v/v) of Tween 80 and 1% (v/v) of ethanol (Vehicle, 2 ml/kg, i.p.) was administered intraperitoneally in normal (Control) or passive cigarette smoking (Smoke) (cigarette smoke exposure at 4%, v/v, smoke/air) rats 1 h before and 2 h after 2,4,6-trinitrobenzene sulfonic acid/ethanol enema. Rats were killed 24 h afterwards to assess the colonic lesion and inflammation. Each value is expressed as mean ± S.E.M., $N = 6–8$/group.

[a] $P < 0.001$, compared with the value of corresponding control vehicle group.
[b] $P < 0.05$, compared with the value of corresponding control vehicle group.
[c] $P < 0.001$, compared with the value of corresponding smoke vehicle group.
[d] $P < 0.05$, compared with the value of corresponding smoke vehicle group.
[e] $P < 0.01$, compared with the value of corresponding smoke vehicle group.

about 70% in the control group (from 9.08 ± 0.8 to 2.8 ± 0.4 ng/mg protein, $P < 0.01$) and about 45% in smoke group (from 8.8 ± 0.3 to 4.8 ± 0.6 ng/mg protein, $P < 0.01$) by indomethacin, respectively, indicating that the colonic cyclooxygenase activity was indeed inhibited by pretreatment with indomethacin at this dosage.

Similarly, pretreatment with nimesulide (25 mg/kg, s.c.) also had a significant protective effect on inflammatory bowel disease induced by 2,4,6-trinitrobenzene sulfonic acid-enema, which was reflected by attenuating the rat body weight loss, the increase of colon weight, as well as lesion area and myeloperoxidase activity in colonic tissue. Moreover, it also had a preventive effect in the cigarette-smoke exposure group (Table 2). It was noted that administration of both indomethacin and nimesulide at these dosages for a 4-day period alone did not show any observable damage to the gastrointestinal tract in rats during the whole experiment. This phenomenon indicated

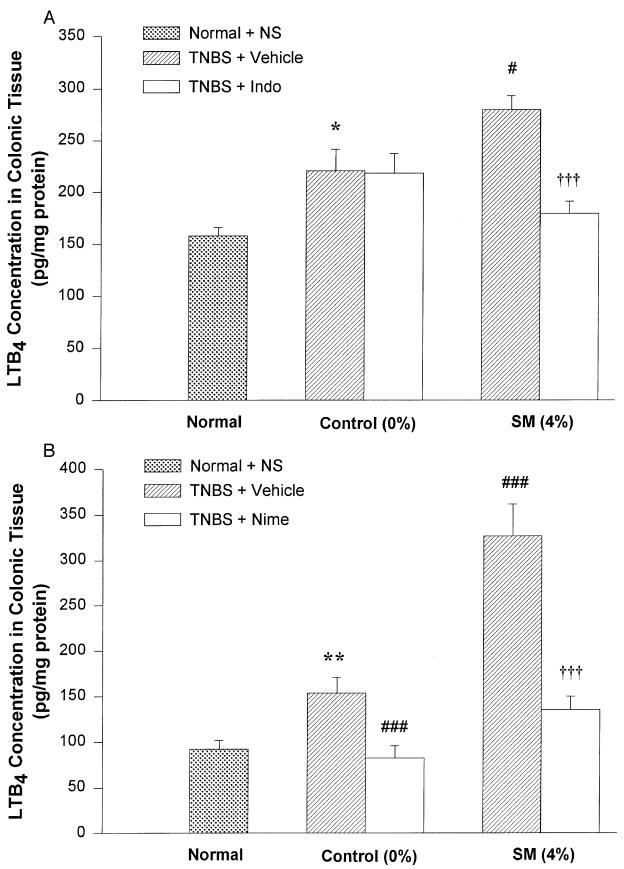

Fig. 1. Effect of pretreatment with (A): indomethacin (Indo, 2.5 mg/kg, s.c.) or (B): nimesulide (Nime, 25 mg/kg, s.c.) together with cigarette smoke (4%, v/v, smoke/air) exposure on LTB_4 concentration in the colonic tissue 24 h after 2,4,6-trinitrobenzene sulfonic acid (TNBS)–ethanol enema in rats. Normal rats were undertaken normal saline (NS) enema, while control (0%) rats were given TNBS–ethanol enema without passive smoking. Smoke (SM) group was exposed to cigarette smoke with TNBS–ethanol enema. Each value is expressed as mean ± S.E.M. of six to eight rats per treatment group. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with normal group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, compared with the control vehicle group; $^{\dagger\dagger\dagger}P < 0.001$, compared with the SM vehicle group.

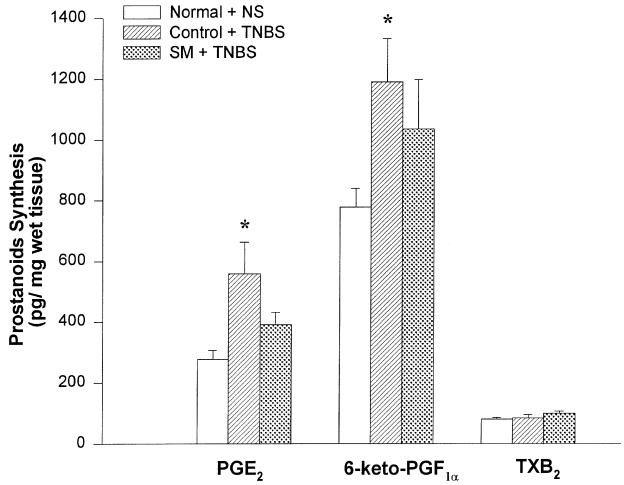

Fig. 2. Effect of cigarette smoke (4%, v/v, smoke/air) exposure on prostanoids synthesis in the colonic tissue ex vivo 24 h after TNBS–ethanol enema in rats. Normal rats were undertaken a NS enema, while control rats were given TNBS–ethanol enema without passive smoking. SM group was exposed to cigarette smoke with TNBS–ethanol enema. Each value is expressed as mean ± S.E.M. of 8–10 rats per treatment group. $^{*}P < 0.05$, compared with the corresponding normal group.

that the two drugs at these doses might not significantly affect the constitutive cyclooxygenase-1 activity, which has a protective role in the gastrointestinal mucosa.

Treatment with SC-236 (2.5 mg/kg, i.p.), a specific cyclooxygenase-2 inhibitor, although only in two doses, also significantly prevented the colonic inflammation and lesion formation induced by 2,4,6-trinitrobenzene sulfonic acid both in control and smoke groups, when compared with the corresponding vehicle group (Table 3). This finding further confirmed that the protective effects of indomethacin, nimesulide as well as SC-236 itself on colonic lesion and inflammation formation is possibly due to the same mechanism inhibiting cyclooxygenase-2 activity.

3.2. Effect of indomethacin and nimesulide on leukotriene B_4 concentration in the colonic tissue

The leukotriene B_4 level in the colonic tissue was significantly enhanced further by pre-exposure of cigarette smoke, which was elevated after 2,4,6-trinitrobenzene sulfonic acid-enema in the control group (Fig. 1). Pretreatment with indomethacin (2.5 mg/kg) could only attenuate the increase of leukotriene B_4 level in the smoke group, but not in the control group with 2,4,6-trinitrobenzene sulfonic acid alone (Fig. 1A). However, the leukotriene B_4 concentration in the colonic tissue was dramatically decreased after nimesulide (25 mg/kg) administration in both control and smoke groups as compared to the corresponding vehicle group (Fig. 1B).

3.3. Effect of cigarette-smoke exposure and nimesulide on prostanoid synthesis from colonic tissue

The synthesis of prostanoids, including prostaglandin E_2, 6-keto-prostaglandin $F_{1\alpha}$ (a stable product of prostacy-

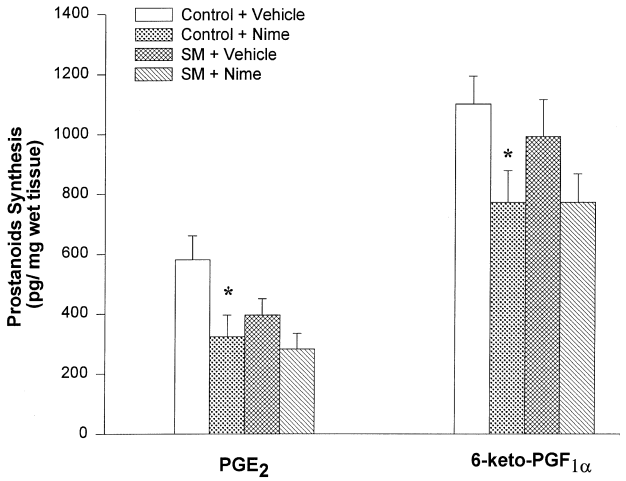

Fig. 3. Effect of nimesulide (Nime, 25 mg/kg, s.c.) pretreatment on prostanoids synthesis in the colonic tissue ex vivo 24 h after TNBS–ethanol enema in rats. Control groups were given TNBS–ethanol enema without passive smoking. SM groups were exposed to cigarette smoke with TNBS–ethanol enema. Each value is expressed as mean ± S.E.M. of 6–8 rats per treatment group. $^{*}P < 0.05$, compared with the corresponding control vehicle group.

clin) and thromboxane B_2 (a stable product of thromboxane A_2) was assayed in colonic tissue 24 h after 2,4,6-trinitrobenzene sulfonic acid–ethanol enema. It was shown that the synthesis of prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$ was significantly increased 24 h after 2,4,6-trinitrobenzene sulfonic acid-enema as compared to the normal tissue. Cigarette-smoke exposure did not impose any further effect on this action; instead, there was a slight decline in prostanoid concentrations. However, the synthesis of thromboxane B_2 was not significantly altered 24 h after 2,4,6-trinitrobenzene sulfonic acid-enema, or did it change after cigarette-smoke exposure (Fig. 2). Pretreatment with nimesulide (25 mg/kg, s.c.) inhibited the increased synthesis of both prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$ in both control and cigarette-smoke exposure groups, although it only had a significant inhibitory effect in the control group, indicating that the colonic cyclooxygenase activity was indeed inhibited by nimesulide pretreatment (Fig. 3). It was noted that the pharmacological intervention by nimesulide on prostanoid concentrations was less in the smoking group. It was probably due to the increase of prostanoid synthesis after 2,4,6-trinitrobenzene sulfonic acid-enema was not as much as the nonsmoking control (Fig. 2), so that nimesulide could produce less pharmacological effect in the smoking group.

3.4. Effect of cigarette-smoke exposure on the expression of cyclooxygenase-1 and cyclooxygenase-2 mRNA and protein in the colonic tissue after 2,4,6-trinitrobenzene sulfonic acid-enema

Using cyclooxygenase-1 and cyclooxygenase-2 specific primer yielded a 398- and 584-base pair product, respec-

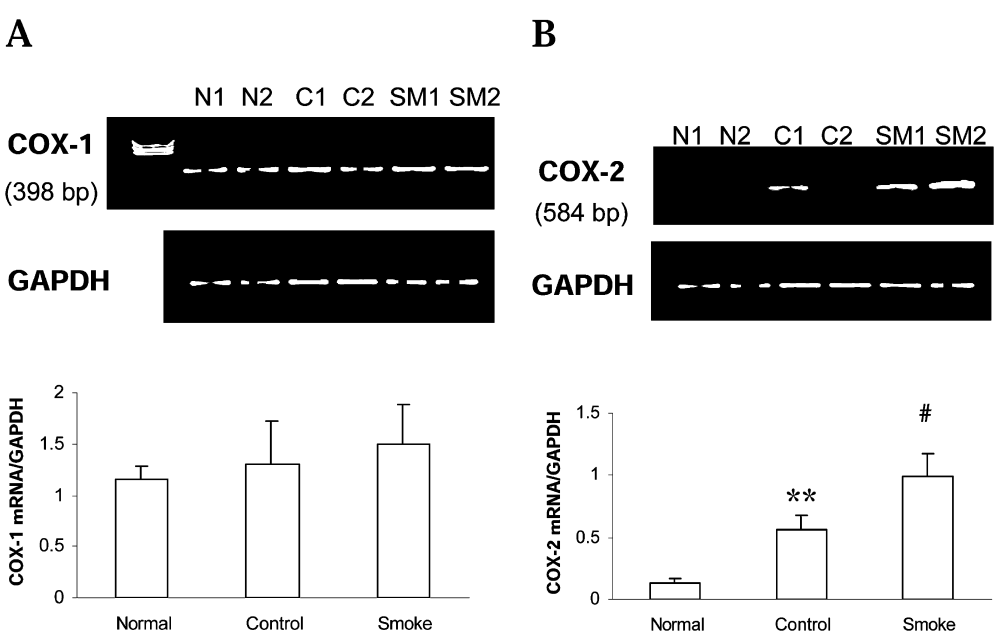

Fig. 4. Effect of cigarette-smoke exposure (4%, v/v, smoke/air) on (A): Cyclooxygenase-1 (COX-1) and (B): Cyclooxygenase-2 (COX-2) mRNA expression in colonic tissue 24 h after TNBS-enema in rats. The gel photographs show the PCR-amplified COX-1 or COX-2 and GAPDH cDNA derived from their respective mRNA. The PCR product fragments of COX-1, COX-2 and GAPDH mRNA are 398, 584 and 309 bp, respectively. N1 and N2 lanes represent for two samples from normal group with normal saline; C1 and C2 lanes represent for two samples from control group with TNBS; SM1 and SM2 lanes represent for two samples from smoke group with TNBS. The gel photographs were scanned with a computerized densitometer. Each value is expressed as mean ± S.E.M. of five to six rats per treatment group. $^{**}P < 0.01$, compared with the normal group; $^{\#}P < 0.05$, compared with the control group.

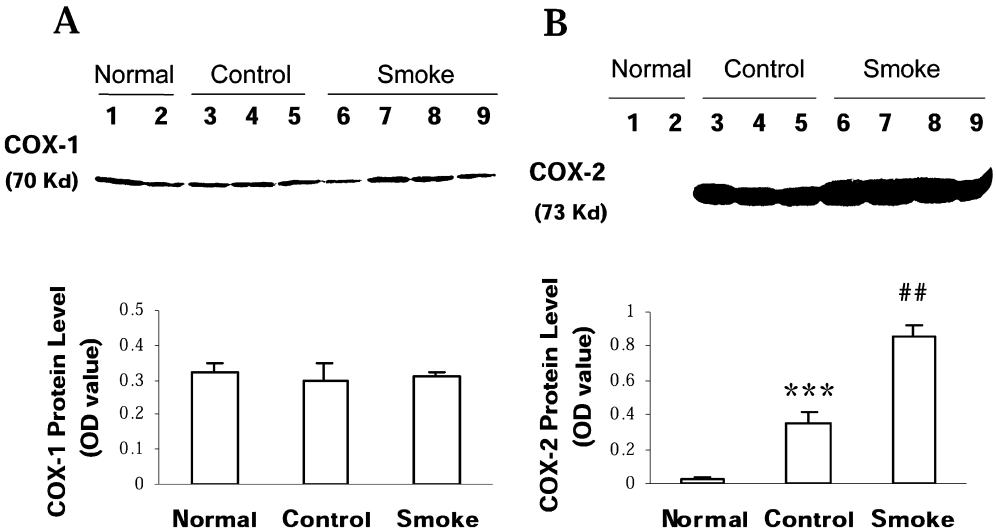

Fig. 5. Effect of cigarette smoke exposure (4%, v/v, smoke/air) on (A): Cyclooxygenase-1 (COX-1) and (B): Cyclooxygenase-2 (COX-2) protein expression in colonic tissue 24 h after 2,4,6-trinitrobenzene sulfonic acid (TNBS)-enema in rats by Western blot analysis. One hundred micrograms of protein lysate was analyzed in each lane. Lanes 1–2 represent for two samples from normal group with normal saline; lanes 3–5 represent for three samples from control group with TNBS; lanes 6–9 represent for four samples from smoke group with TNBS. The film photographs were scanned with a computerized densitometer expressing by optical density value. Each value is expressed as mean ± S.E.M. of six to seven rats per treatment group. $^{***}P < 0.001$, compared with the normal group; $^{\#\#}P < 0.01$, compared with the control group.

tively, identifying cyclooxygenase-1 and cyclooxygenase-2 mRNA expression in the colonic tissue (Fig. 4A and B). Samples from normal, 2,4,6-trinitrobenzene sulfonic acid control and smoke with 2,4,6-trinitrobenzene sulfonic acid groups showed a similar signal for cyclooxygenase-1 mRNA (Fig. 4A), while cyclooxygenase-2 mRNA only highly expressed in the tissue from 2,4,6-trinitrobenzene sulfonic acid group (Fig. 4B). Moreover, it was shown that the signals of cyclooxygenase-2 from the smoke with 2,4,6-trinitrobenzene sulfonic acid group were much denser than those from the control group (Fig. 4B). Hence, it was indicated that pretreatment with cigarette smoke could intensify the cyclooxygenase-2 gene expression in the tissue 24 h after 2,4,6-trinitrobenzene sulfonic acid–ethanol enema.

Western blotting analysis of crude homogenates of colonic tissue reflected a similar trend of cyclooxygenase protein expression as its gene expression in the colonic tissue (Fig. 5). It was shown that cyclooxygenase-1 protein expressions (a specific 70-kDa band) in normal, 2,4,6-trinitrobenzene sulfonic acid control and smoke with 2,4,6-trinitrobenzene sulfonic acid groups were indifferent (Fig. 5A), indicating that cyclooxygenase-1 protein was constitutively expressed in the colonic tissue and was not significantly changed after 2,4,6-trinitrobenzene sulfonic acid-enema or cigarette-smoke exposure. In contrast, cyclooxygenase-2 protein (a specific 73-kDa band) was only highly expressed 24 h after 2,4,6-trinitrobenzene sulfonic acid-enema, and it was significantly augmented further by pretreatment with cigarette-smoke exposure (Fig. 5B). In addition, a time-course study showed that cyclooxygenase-2 protein expression could be detected as early as 6 h after 2,4,6-trinitrobenzene sulfonic acid-enema, and peaked at 24 h (data not shown), indicating that the cyclooxygenase-2 protein expression could be induced at the very early stage of colonic lesion and inflammation formation after 2,4,6-trinitrobenzene sulfonic acid-enema. Immunostain data showed that cyclooxygenase-2 protein was predominantly expressed in the smooth muscle, infiltrating cells in the submucosa, and submucosal blood vessels (data not shown).

4. Discussion

The present study confirmed our previous finding that passive cigarette smoking indeed aggravated the acute colonic lesion and inflammation formation induced by 2,4,6-trinitrobenzene sulfonic acid in rats (Guo et al., 1999, 2000). Moreover, using the same inflammatory bowel disease model, the present study clearly identified that pretreatment with indomethacin, a nonselective cyclooxygenase-2 inhibitor, and nimesulide, a moderately selective cyclooxygenase-2 inhibitor, not only protected against the colonic lesion and inflammation induced by 2,4,6-trinitrobenzene sulfonic acid, but also considerably attenuated the exacerbation provoked by cigarette-smoke exposure. Furthermore, the expression of mRNA and protein of cyclooxygenase-2 but not cyclooxygenase-1 was clearly identified to be highly induced after 2,4,6-trinitrobenzene sulfonic acid-enema and it was potentiated further by cigarette-smoke exposure. Administration of a specific cyclooxygenase-2 inhibitor SC-236 produced a similar protective effect as in indomethacin and nimesulide. It was

therefore strongly indicated that the protective role of cyclooxygenae-2 inhibitors in the colonic damage induced by 2,4,6-trinitrobenzene sulfonic acid was largely due to the inhibition of the highly induced cyclooxygenase-2 activity after 2,4,6-trinitrobenzene sulfonic acid-enema. It was also suggested that the potentiation of cyclooxygenase-2 expression by cigarette-smoke exposure during the initiation process of colonic lesion and inflammation formation induced by 2,4,6-trinitrobenzene sulfonic acid contributed at least in part to the aggravating effect of cigarette-smoke exposure on this experimental inflammatory bowel disease model.

It is known that cyclooxygenase-2 is an inducible form of cyclooxygenase, which is a rate-limiting enzyme in prostanoids synthesis from arachidonic acids (Crofford, 1997). As a result of highly expressed cyclooxygenase-2 protein, the synthesis of prostaglandins (prostaglandin E_2 and prostacyclin) was increased 24 h after 2,4,6-trinitrobenzene sulfonic acid-enema. However, it was not further enhanced by pre-exposure to cigarette smoke although the cyclooxygenase-2 protein expression was elevated by cigarette-smoke exposure, suggesting that there might be a mechanism operating between the cyclooxygenase-2 protein expression and its catalyzing activity in the production of prostaglandins, which could be affected by cigarette-smoke exposure. Indeed, it had been reported that the decreased level of antioxidant-glutathione in the tissue resulted in prostaglandin E_2 synthesis inhibition when exposed to oxidant stress (Hempel and Wessels, 1994; Wentzel et al., 1999). Reduced glutathione (GSH) level in the colonic tissue was found to be significantly reduced by cigarette-smoke exposure after 2,4,6-trinitrobenzene sulfonic acid-enema (Guo et al., 2000). This might explain why there was no further increase in prostaglandin synthesis although there had been a marked increase of cyclooxygenase-2 protein expression in the colonic tissue by cigarette-smoke exposure. In addition, it had been shown that nicotine given in doses to produce similar blood nicotine concentration as in heavy smokers suppressed cyclooxygenase products in rectal mucosa of rabbits (Zijlstra et al., 1994). In our previous study, under the similar experimental condition as shown in the present study, the nicotine concentration was similarly achieved in rats after cigarette smoking (Chow et al., 1997). This concentration of nicotine could inhibit prostanoid synthesis in the colon in spite of increased expression of cyclooxygenase-2 after cigarette-smoke exposure. Nevertheless, the potentiation of cyclooxygenase-2 expression by cigarette-smoke exposure may contribute at least in part to the adverse action of passive cigarette smoking on 2,4,6-trinitrobenzene sulfonic acid-induced inflammatory bowel disease in rats, although it seems not to be due to further increase in prostaglandin synthesis in the colonic tissue.

The contribution of high expression of cyclooxygenase-2 to the formation of colonic lesion and inflammation may be due to its prostaglandin products, such as prostaglandin E_2. However, thromboxane A_2 (which was not significantly changed after 2,4,6-trinitrobenzene sulfonic acid-enema) seemed not to be involved in the acute stage of lesion and inflammation formation induced by 2,4,6-trinitrobenzene sulfonic acid, which is consistent with a previous finding (Rachmilewitz et al., 1989). On the other hand, previous studies found that prostaglandin E_2 as well as other arachidonic acids products by the cyclooxygenase pathway were markedly increased after 2,4,6-trinitrobenzene sulfonic acid-enema, and had a greater importance in the acute phase of the inflammatory response induced by 2,4,6-trinitrobenzene sulfonic acid compared to the later chronic phase (Boughton-Smith et al., 1988; Wallace et al., 1992). It is well known that prostaglandin E_2 is an important inflammatory mediator involved in many inflammation processes and diseases. It induces epithelial cell chloride secretion, which results in diarrhea observed in inflammatory bowel disease patients (Weymer et al., 1985). The diarrhea may lead to body weight loss during inflammatory bowel disease. So, in the present study, the marked attenuation of rat body weight loss in 2,4,6-trinitrobenzene sulfonic acid-induced inflammatory bowel disease model by pretreatment with the cyclooxygenase-2 inhibitors might be the result of inhibition of prostaglandin E_2 synthesis in the colonic tissue. This may also explain why passive cigarette smoking did not adversely affect the body weight loss, as it did not increase prostaglandin E_2 synthesis further in 2,4,6-trinitrobenzene sulfonic acid-treated animals. Prostaglandin E_2 also acts as a vasodilator in blood vessels, which could induce mucosal hyperemia and may lead to hemorrhagic lesion (Brown and Zipser, 1987). Moreover, an in vitro study also clearly demonstrated that pretreatment with indomethacin had a protective action on the injury of human colonic cells induced by incubation with 2,4,6-trinitrobenzene sulfonic acid, which was accompanied with an inhibition of prostaglandins synthesis (Stratton et al., 1996). These findings are in accord with the present study, in which inhibition of prostaglandins synthesis by the cyclooxygenase-2 inhibitors resulted in attenuation of the inflammatory responses provoked by 2,4,6-trinitrobenzene sulfonic acid-enema.

However, there have been reports suggesting that nonsteroidal anti-inflammatory drugs (NSAIDs) increase the level of clinical activity and worsen the symptoms in inflammatory bowel disease patients (Eberhart and Dubois, 1995). In animal studies, there are still controversial findings about the effects of treatment with indomethacin or other cyclooxygenase-2 inhibitors on experimental inflammatory bowel disease models. Some reported that indomethacin administration decreased the inflammatory responses (Mann and Demers, 1983); others showed no effect or even exacerbation of the healing process of inflammatory bowel disease in rats (Boughton-Smith et al., 1988; Wallace et al., 1992; Reuter et al., 1996). The reason for the conflicting findings may be due to the different

time of drug administration. The present study reported for the first time that cyclooxygenase-2 inhibitors given beforehand protected against colitis, which was different from the previous studies in which the drugs were administered after the experimental induction of inflammatory bowel disease. Based on these findings, it is suggested that inhibition of prostaglandin synthesis, which may come mainly from the highly induced cyclooxygenase-2 expression rather than cyclooxygenase-1, may be beneficial to the initiation of inflammation in the colonic tissue. In contrast, blocking the cyclooxygenase-2 activity could be detrimental to the healing process after the lesion and inflammation establishment in the colon, since proliferating activity of prostaglandins (Uribe et al., 1992) would be blunted by the cyclooxygenase inhibitors, which contributes to the healing process in inflammatory bowel disease. Moreover, the present results were consistent with the previous clinical findings that cyclooxygenase-2 protein and mRNA were only highly expressed in the most affected area in the active inflammatory bowel disease patients, which indicated that cyclooxygenase-2 was involved in the acute inflammatory response of chronic inflammatory bowel disease (Hendel and Nielsen, 1997; Singer et al., 1998). The present finding may also give direct evidence to understand the pathogenesis of inflammatory bowel disease and open up new field for developing therapeutic approaches.

It was also noted that the potentiation of leukotriene B_4 production and the myeloperoxidase activity in the colonic tissue by cigarette-smoke exposure was almost abolished by both indomethacin and nimesulide administration. It is well known that leukotriene B_4 is a potent chemotactic agent for stimulating neutrophil infiltration and degranulation in the tissue during ulceration and inflammation (Zimmerman et al., 1990; Wallace and Keenan, 1990). Furthermore, it was shown to play an important role in the pathogenesis of inflammatory bowel disease in humans (Sharon and Stenson, 1984) as well as in animals (Wallace et al., 1989). On the other hand, it has been demonstrated that neutrophil infiltration and activation, which is marked by myeloperoxidase activity in the tissue, play essential roles in the pathogenesis of experimental inflammatory bowel disease models (Domek et al., 1995; Wallace et al., 1998). These observations suggest that the attenuating effect of indomethacin and nimesulide on the adverse action of cigarette-smoke exposure on the 2,4,6-trinitrobenzene sulfonic acid-induced inflammatory bowel disease may partially be due to the inhibition of neutrophil infiltration and leukotriene B_4 production in the colonic tissue, which were potentiated further by cigarette-smoke exposure rather than direct suppression of prostaglandins synthesis. However, how cyclooxygenase-2 inhibitors could attenuate colonic leukotriene B_4 production and myeloperoxidase activity is still not known.

Taken together, the present study for the first time demonstrated that pretreatment with cyclooxygenase-2 inhibitors could significantly protect the acute colonic damage formation induced by 2,4,6-trinitrobenzene sulfonic acid, and could also markedly attenuate the adverse action of passive cigarette smoking on the initiation of colonic damage in this inflammatory bowel disease model. These protective effects may likely be due to the inhibitory effect on cyclooxygenase-2 activity, which was highly induced after 2,4,6-trinitrobenzene sulfonic acid-enema. In contrast to the protective role in the healing process in inflammatory bowel disease, the high expression of cyclooxygenase-2 in the initiating stage of experimental inflammatory bowel disease seems to act as a promoter of the colonic lesion and inflammation formation. Furthermore, the potentiation of cyclooxygenase-2 expression by cigarette-smoke exposure may contribute at least in part to the adverse action of cigarette-smoke exposure on the pathogenesis of the experimental inflammatory bowel disease in rats.

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