Induction of Small Intestinal Damage in Rats Following Combined Treatment with Cyclooxygenase-2 and Nitric-Oxide Synthase Inhibitors

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Received January 21, 2004; accepted March 24, 2004

ABSTRACT

Nitric oxide (NO) produced by constitutively expressed NO synthase (cNOS) plays an important role in maintaining the mucosal integrity of the small intestine, in collaboration with prostaglandins produced by cyclooxygenase (COX)-1. We examined whether intestinal damage is provoked in rats under inhibition of both cNOS and COX-2. The animals were given L-NAME (N\textsuperscript{G}-nitro-L-arginine methyl ester), aminoguanidine, or rofecoxib, either alone or in combination, and killed 24 h later. Neither L-NAME nor aminoguanidine alone provoked damage in the small intestinal mucosa within 24 h, yet L-NAME produced damage in a l-arginine-sensitive manner when administered together with rofecoxib. L-NAME up-regulated the expression of COX-2 mRNA, and the prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) content following the L-NAME administration significantly increased 12 h later, in both a rofecoxib- and a l-arginine-inhibitable manner. L-NAME enhanced intestinal motility, decreased mucus secretion, and increased the number of bacteria in the mucosa. The up-regulation of COX-2 expression and PGE\textsubscript{2} production by l-NAME was inhibited by prior administration of atropine, at a dose that inhibited the intestinal hypermotility. The intestinal lesions induced by l-NAME plus rofecoxib were prevented by pretreatment with ampicillin and aminoguanidine as well as atropine, indicating the involvement of bacteria, inducible nitric oxide synthase, and hypermotility in the pathogenesis. These results suggest that inhibition of both cNOS and COX-2 provokes intestinal damage, similar to inhibition of both COX-1 and COX-2. Inhibition of cNOS, similar to COX-1, up-regulates COX-2 expression, the process being associated with intestinal hypermotility and bacterial invasion, and this may be a key to the occurrence of intestinal damage associated with COX-2 inhibition.

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin cause gastrointestinal ulceration as an adverse reaction (Whittle, 1981; Lanza, 1984; Takuechi et al., 1986). Although a deficiency of prostaglandins (PGs) caused by cyclooxygenase-1 (COX-1) is considered to play a critical role in the pathogenesis of these lesions (O’Neill and Ford-Hutchinson, 1993), recent studies showed that inhibition of both COX-1 and COX-2 is required for induction of damage in the gastrointestinal mucosa following administration of NSAIDs (Wallace et al., 2000; Tanaka et al., 2001). Since inhibition of COX-1 was accompanied by the expression of COX-2 mRNA in these tissues (Tanaka et al., 2002a,b) and mucosal PGE\textsubscript{2} levels recovered from 6 h after administration of SC-560 in a rofecoxib-sensitive manner (Tanaka et al., 2002b), it is assumed that PGs produced by COX-2 contribute to maintaining the mucosal integrity of the gastrointestinal tract by antagonizing deleterious events caused by inhibition of COX-1.

Nitric oxide (NO) plays a dual role in the pathogenesis of NSAID-induced intestinal damage: a protective role when produced by constitutively expressed NO synthase (cNOS) and a pro-ulcerogenic role when produced by inducible NO synthase (iNOS) (Tanaka et al., 1999). Indeed, indomethacin-induced intestinal lesions were prevented or aggravated by prior administration of NOR-3, the NO donor, or l-NAME (N\textsuperscript{G}-nitro-l-arginine methyl ester), the nonselective NOS inhibitor, respectively (Tanaka et al., 2001). Thus, NO produced by cNOS is important for maintaining the mucosal integrity of the small intestine, just mimicking the role of COX-1/PGs. If so, intestinal damage might also be induced by inhibition of both cNOS and COX-2.
In the present study, we therefore examined whether or not intestinal damage is provoked in rats following the combined administration of 1-NAME and the selective COX-2 inhibitor rofecoxib (Chan et al., 1995). We also examined whether inhibition of NO production by 1-NAME up-regulates the expression of COX-2 mRNA in the intestinal mucosa, similar to inhibition of PGs by SC-560, the selective COX-1 inhibitor (Smith et al., 1998). In addition, since NSAID-induced intestinal lesions occur in association with various functional events, such as intestinal motility and bacterial counts (Tanaka et al., 2002c), we investigated the effect of COX and NOS inhibitors on these parameters at different time points, according to a previous study (Takeuchi et al., 2002).

**Materials and Methods**

**Animals.** Male Sprague-Dawley rats (220–260 g; Nippon Charles River Laboratories, Inc., Shizuoka, Japan) were used. Studies were carried out using groups of four to eight animals without fasting, unless otherwise specified. All experimental procedures described here were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

**Evaluation of Small Intestinal Ulcerogenic Properties.** In the first study, the animals were given p.o. with SC-560 (a selective COX-1 inhibitor: 10 mg/kg) (Smith et al., 1998; Tanaka et al., 2002b), rofecoxib (a selective COX-2 inhibitor: 10 mg/kg) (Chan et al., 1995; Tanaka et al., 2002b), or SC-560 plus rofecoxib. In the second study, they were given s.c. with 1-NAME (10, 20, and 50 mg/kg) or aminoguanidine (20 mg/kg), together with or without p.o. administration of rofecoxib (10 mg/kg). The dose of 1-NAME or aminoguanidine was selected to inhibit basal NO production or iNOS activity in rats, respectively (Tanaka et al., 1999, 2001). In the case of 1-NAME (50 mg/kg) plus rofecoxib, the protective effect of L-arginine (500 mg/kg), atropine (3 mg/kg; an anticholinergic drug), ampicillin (800 mg/kg; an antibiotic), or aminoguanidine (20 mg/kg) on the intestinal lesions was examined. L-arginine, atropine, and aminoguanidine were given s.c. 30 min before administration of 1-NAME plus rofecoxib, whereas ampicillin was given p.o. twice 24 h and 30 min before. In each study, the animals were killed 24 h later under deep diethyl ether anesthesia, and the small intestine was excised and treated with 2% formalin for fixation of the tissue walls. Then, it was opened along the antimesenteric attachment and examined for damage under a dissecting microscope with square grids (×10). The area (millimeters squared) of macroscopically visible lesions was measured, summed per small intestine, and used as a lesion score. The person measuring the lesions did not know the treatments given to the animals. In some cases, the animals were killed at various time points (3, 6, 12, and 24 h) after administration of treated with 1-NAME plus rofecoxib.

**Determination of Mucosal PGE2 Content.** Levels of PGE2 in the small intestinal mucosa were measured after administration of SC-560 (10 mg/kg), rofecoxib (10 mg/kg), L-NAME (50 mg/kg), or aminoguanidine (20 mg/kg). The animals were killed under deep diethyl ether anesthesia 3 h after the administration, and the small intestinal tissue was isolated, weighed, and placed in a tube containing 100% ethanol plus 0.1 M indomethacin (Putaki et al., 1994). In some cases, the mucosal PGE2 content was measured at 12 h after administration of L-NAME (50 mg/kg), with or without rofecoxib, L-arginine (500 mg/kg; s.c.), or atropine (3 mg/kg; s.c.) given 30 min before L-NAME. Then, the tissues were homogenized using a polytron homogenizer (IKA, Tokyo, Japan) and centrifuged at 10,000 rpm for 10 min at 4°C. After the supernatant of each sample had been evaporated with N2 gas, the residue was dissolved in assay buffer and used for determination of PGE2. The concentration of PGE2 was measured using a PGE2 enzyme immunoassay kit (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK).

**Analysis of COX-2 and Glyceraldehyde-3-Phosphate Dehydrogenase (G3PDH) mRNA Expression by Reverse Transcription-PCR.** The animals were killed under deep diethyl ether anesthesia 6 h after administration of SC-560 (10 mg/kg), rofecoxib (10 mg/kg), or L-NAME (50 mg/kg), and their small intestines were removed, frozen in liquid nitrogen, and stored at −80°C until use. In some cases, L-NAME was administered together with L-arginine (500 mg/kg, s.c.) or atropine (3 mg/kg, s.c.) given 30 min before L-NAME. Intestinal tissue samples were pooled from two to three rats for extraction of total RNA, which was prepared by a single-step acid phenol-chloroform extraction procedure by use of TRIzol (Invitrogen, Carlsbad, CA). Total RNA primed by random hexadeoxyribonucleotide was reverse-transcribed with the SuperScript preamplification system (Invitrogen). The sequences of sense and antisense primers for the rat COX-2 were 5′-ATATGACTGCCCACACTCCCATG-3′ and 5′-AATTGTGAAG GTGCCGGACGC-3′, respectively, giving rise to a 702-base pair PCR product (Iso et al., 1995). For the rat G3PDH, a housekeeping gene, the sequences were 5′-GAACGGGAACCTCATTGACCATGGCC-3′ for the sense primer and 5′-TGGAGGCCACAC-CTGTTGCTGT-3′ for the antisense primer, giving rise to a 310-base pair PCR product (Feng et al., 1993). An aliquot of the reverse transcription reaction product served as a template in 35 cycles of PCR with 1 min of denaturation at 94°C, 0.5 min of annealing at 58°C, and 1 min of extension at 72°C on a thermal cycler. A portion of the PCR mixture was electrophoresed in 1.8% agarose gel in Tris-EDTA-acetic acid buffer (40 mM Tris buffer, 2 mM EDTA, and 20 mM acetic acid, pH 8.1), and the gel was stained with ethidium bromide and photographed.

**Determination of Small Intestinal Motility.** Intestinal motility was determined using a miniature balloon according to previous papers (Tanaka et al., 2002c; Takeuchi et al., 2002). In brief, the rat was anesthetized with urethane (1.25 g/kg i.p.), and the trachea was cannulated to facilitate respiration. A midline incision was made to expose the small intestine, and a thin, saline-filled balloon, made from silicone rubber and attached to a polyethylene catheter, was introduced into the jejunum via a small incision and tied in place avoiding large blood vessels. The volume in the balloon was adjusted to give an initial resting pressure of 5 mm Hg, which was not sufficient to cause active distension of the intestinal wall, and after allowing the preparation to rest for 30 min, intestinal motility was monitored on a recorder (U-228; Tokai-Irika, Tokyo, Japan) as intraluminal pressure changes, through a pressure transducer and polygraph recorder (Nihon Koden, Ibaragi, Japan). SC-560 (10 mg/kg), rofecoxib (10 mg/kg), or L-NAME (50 mg/kg) was given i.d. or s.c., respectively, after basal intestinal motility had stabilized, and the motility was measured for 2 h thereafter. In some cases, the effect of L-arginine (500 mg/kg; s.c.) or atropine (3 mg/kg s.c.) on the intestinal hypermotility induced by 1-NAME was also examined.

**Determination of the Number of Enterobacteria.** The enterobacteria were enumerated according to a modified method originally described by Reuter et al. (1997). Six hours after administration of SC-560 (10 mg/kg), rofecoxib (10 mg/kg), or L-NAME (50 mg/kg), the animals were killed under deep diethyl ether anesthesia, and their small intestine was removed. After the rinsing of each intestine with sterile saline, the mucosa was scraped with glass slides, weighed, and homogenized in 1 ml of sterile phosphate-buffered saline (PBS) per 100 mg of wet tissue. Aliquots of the homogenate were plated on blood agar and Gifu anaerobic medium agar (Nisui, Tokyo, Japan). Blood agar plates were incubated at 37°C for 24 h under aerobic conditions, whereas Gifu anaerobic medium agar plates were incubated for 48 h under standard anaerobic conditions (BBL Gas Pack Pouch Anaerobic System; BD Biosciences, San Jose, CA). Plates containing 10 to 200 colony-forming units (CFU) were examined for numbers of enterobacteria, and the data were expressed as log CFU per gram of tissue.

**Determination of Intestinal Mucus Content.** Two hours after administration of various COX and NOS inhibitors, the animals were killed under deep diethyl ether anesthesia, and the intestine
was removed and opened along the antimesenteric attachment. The mucous content including soluble and adherent mucus gel was measured as described by Azumi et al. (1981). Briefly, the intestinal mucosa was immersed in 20 ml of PBS containing 2% (w/w) N-acetyl-D-glucosamine and gently stirred with a magnetic stirrer for 5 min at room temperature. Then, the mucosa was rinsed with 10 ml of PBS twice, and the solution recovered as the adherent samples was dissolved in 4 ml of 50 mM Tris-HCl (pH 7.2) containing 2% Triton X-100, homogenized with a Polytron tissue homogenizer (IK), and centrifuged at 10,000 rpm for 30 min at 4°C. An aliquot of the supernatant was applied to a column of Sepharose 2B (Amersham Biosciences AB, Uppsala, Sweden) every 50 cm and eluted with 50 mM Tris-HCl (pH 7.2) containing 2% Triton X-100. The void volume fraction was collected, and the hexose content was measured as mucin by the phenol-sulfuric acid method, using galactose as the standard.

**Preparation of Drugs.** The drugs used were SC-560 (Cayman Chemical, Ann Arbor, MI), rofecoxib (Banyu, Tokyo, Japan), L-NAME, aminoguanidine, L-arginine, atropine (Nakarai tesque, Chemical, Ann Arbor, MI), rofecoxib (Banyu, Tokyo, Japan), and urethane (Tokyo Kasei, Tokyo, Japan). Both SC-560 and rofecoxib were suspended in a hydroxy propyl cellulose solution (Wako Pure Chemicals, Osaka, Japan), whereas other drugs were dissolved in saline. All drugs were prepared immediately before use and administered p.o., i.d., or s.c. in a volume of 0.5 ml/100 g body weight.

**Statistics.** Data are presented as the mean ± S.E. of four to eight rats per group. Statistical analyses were performed using the two-tailed Dunnett’s multiple comparison test, and values of P < 0.05 were considered significant.

**Results**

**Effects of COX and NOS Inhibitors on Intestinal Mucosa and PGE2 Content.** Neither SC-560 (10 mg/kg) nor rofecoxib (10 mg/kg) induced damage in the small intestine within 24 h (Fig. 1A); however, when SC-560 was given together with rofecoxib, this treatment produced hemorrhagic lesions at an incidence of 100%, the lesion score being 89.1 ± 7.2 mm². Likewise, L-NAME, the nonselective NOS inhibitor (50 mg/kg), did not by itself cause any damage in the intestine, yet the agent provoked damage when given together with rofecoxib, the selective COX-2 inhibitor, the damage score being 23.1 ± 5.2 mm² (Fig. 1B). The ulcerogenic action of L-NAME in combination with rofecoxib was observed in a dose-dependent manner, and the damage score was 4.6 ± 2.3 and 9.2 ± 3.1 mm² at 10 and 20 mg/kg, respectively (data not shown). Following administration of L-NAME plus rofecoxib, the intestinal lesions were first observed from 6 h later, slightly increased 12 h later, and reached maximum 24 h later, the damage score being 0.7 ± 0.5, 2.0 ± 0.8, 5.5 ± 1.3, and 25.1 ± 5.7 mm², respectively. In contrast, aminoguanidine, the selective iNOS inhibitor (20 mg/kg s.c.), did not cause damage, irrespective of whether or not the agent was given together with rofecoxib. Since L-NAME is known to produce a marked increase in blood pressure in rats (Takeuchi et al., 1994), it is possible that the intestinal ulcerogenic response observed in the present study is due to such increases in blood pressure. To check this point, we examined whether intestinal damage was induced in the presence of rofecoxib (10 mg/kg) by administration of norepinephrine at the dose (1 mg/kg, s.c.) that increased arterial blood pressure similar to L-NAME; however, this treatment caused only minimal change in the intestinal mucosa, and the lesion score was 1.5 ± 1.4 mm², which is not significantly different from that (1.9 ± 0.4 mm²) in animals given saline alone.

On the other hand, SC-560 markedly decreased the mucosal PGE2 content of the small intestine from 24.8 ± 4.0 ng/g tissue to less than 2 ng/g tissue within 3 h, whereas rofecoxib had no effect on the PGE2 content (Fig. 2). Neither L-NAME nor aminoguanidine had any effect on the mucosal PGE2 content of the small intestine when determined 3 h after the administration.

**Fig. 1.** Intestinal ulcerogenic responses induced in rats by SC-560, L-NAME or aminoguanidine alone and in the presence of rofecoxib. A, the animals were administered with SC-560 (10 mg/kg) and rofecoxib (10 mg/kg) p.o., either alone or in combination, and they were killed 24 h later. B, the animals were administered L-NAME (50 mg/kg) and aminoguanidine (20 mg/kg s.c.), and the intestinal mucosa was examined 24 h later. One-half the animals were given rofecoxib (10 mg/kg) p.o. 30 min after L-NAME or aminoguanidine. Data are presented as the mean ± S.E. from four to seven rats. SC, SC-560; Rof, Rofecoxib.

**Fig. 2.** Effect of COX and NOS inhibitors on early measurement of mucosal PGE2 content of the rat small intestine. Animals given SC-560 (10 mg/kg p.o.), rofecoxib (10 mg/kg p.o.), L-NAME (50 mg/kg s.c.), or aminoguanidine (20 mg/kg s.c.), were killed 3 h later, and the PGE2 content was measured by enzyme immunoassay. Data are presented as the mean ± S.E. from five to seven rats. *, significant difference from control (saline p.o.) at P < 0.05.
Effect of COX and NOS Inhibitors on Bacterial Invasion.
The number of aerobic and anaerobic enterobacteria in the normal intestinal mucosa was $6.86 \pm 0.15$ and $7.14 \pm 0.11$ log CFU/g tissue, respectively (Fig. 3). Six hours following the administration of SC-560, both values were markedly increased as compared with controls, being $8.24 \pm 0.21$ and $8.25 \pm 0.18$ log CFU/g tissue, respectively. Likewise, L-NAME also increased the number of bacteria in the mucosa, the values for both aerobic and anaerobic bacteria being equivalent to those observed in SC-560-treated animals; however, the bacterial count remained unchanged following administration of rofecoxib or aminoguanidine.

Effects of Ampicillin, Aminoguanidine, L-Arginine, and Atropine on Intestinal Lesions Induced by L-NAME Plus Rofecoxib.
The combined treatment with L-NAME plus rofecoxib caused the increase in mucosal bacterial invasion, similar to indomethacin at the ulcerogenic dose (Tanaka et al., 1999; Kunikata et al., 2002; Takeuchi et al., 2002). In the latter model, the bacterial invasion plays a pathogenetic role in the overproduction of NO through iNOS induction (Tanaka et al., 1999). Thus, we further investigated, using ampicillin the antibiotics and aminoguanidine the selective inhibitor of iNOS, whether the same would be applied to the pathogenesis of intestinal lesions in response to L-NAME plus rofecoxib. As shown in Fig. 4, the intestinal lesions induced by L-NAME plus rofecoxib were significantly attenuated by prior administration of ampicillin (800 mg/kg p.o.) as well as aminoguanidine (20 mg/kg s.c.), the inhibition being over 90% in both cases. Likewise, the intestinal ulcergenic response induced by L-NAME plus rofecoxib was significantly attenuated by prior administration of L-arginine (500 mg/kg, s.c.) or atropine (3 mg/kg, s.c.), and the lesion score in both cases decreased to less than 5 mm$^2$.

Expression of COX-2 mRNA in the Intestinal Mucosa.
The gene expression of COX-2 was negligible in the small intestine of normal rats; however, it was clearly up-regulated 6 h after administration of L-NAME (50 mg/kg) or SC-560 (10 mg/kg) but not rofecoxib (10 mg/kg) (Fig. 5A). By contrast, G3PDH mRNA was observed in the intestinal mucosa, irrespective of whether the animal was treated with vehicle, L-NAME, SC-560, or rofecoxib. In addition, the expression of COX-2 mRNA following treatment with L-NAME was curtailed when the animal was pretreated with L-arginine (500 mg/kg s.c.) or atropine.

Changes in Mucosal PGE$_2$ Content at a Later Period.
Although SC-560 at 10 mg/kg significantly decreased the mucosal PGE$_2$ content at 3 h after the administration, this effect was reversed by PG production due to up-regulation of COX-2 expression 12 h later (Tanaka et al., 2002b). Since we discovered in the present study that L-NAME up-regulated COX-2 expression similar to SC-560, the same might be
observed in the animals treated with L-NAME. Thus, we measured the mucosal PGE₂ content 12 h after the administration of L-NAME, in the absence or presence of rofecoxib.

The mucosal PGE₂ content of the small intestine was significantly increased by L-NAME (50 mg/kg) when determined at 12 h after the administration (Fig. 6); however, the increase in PGE₂ content induced by L-NAME was significantly attenuated by the combined administration of rofecoxib (10 mg/kg) and L-arginine (500 mg/kg) as well as atropine (3 mg/kg). The PGE₂ content observed at 12 h after administration of SC-560 remained at the same levels as control, yet the values significantly decreased on the combined administration of rofecoxib.

Effects of COX and NOS Inhibitors on Intestinal Mucous Content. The total amount of mucus in the small intestine under basal conditions was 7.9 ± 1.2 µg galactose/cm tissue. The mucus content significantly decreased in response to SC-560 (10 mg/kg), reaching 5.2 ± 0.6 µg galactose/cm tissue 3 h later, approximately 65.8% of basal values (Fig. 7). Likewise, s.c. administration of L-NAME (50 mg/kg) also significantly decreased the secretion of mucus (4.8 ± 1.5 µg galactose/cm tissue). Neither rofecoxib (10 mg/kg) nor aminoguanidine (20 mg/kg) had any effect on intestinal mucus secretion.

Effects of COX and NOS Inhibitors on Intestinal Motility. Since intestinal hypermotility has been implicated as one of the pathogenetic factors in NSAID-induced small intestinal lesions (Takeuchi et al., 2002), we examined the effects of various COX inhibitors on intestinal motility. Under urethane anesthesia, no clear contraction was observed in the small intestine of normal rats, resulting in a fluctuation at baseline levels; however, the intestinal motility was markedly enhanced following administration of SC-560, in regard to both the amplitude and frequency of contraction (Fig. 8A). L-NAME also caused intestinal hypermotility, similar to SC-560, and the effect persisted for over 2 h. In contrast, rofecoxib did not have any influence on intestinal motility, and no clear contraction was observed before and after administration of this agent. Likewise, aminoguanidine had no effect on intestinal motility (data not shown). The increased motility caused by L-NAME was potently inhibited by L-arginine (500 mg/kg s.c.) or atropine (3 mg/kg s.c.); this inhibiting effect was given 1 h after the administration of L-NAME.

Discussion

The present study confirmed our previous finding that neither SC-560 nor rofecoxib alone caused intestinal damage,
yet their combination provoked gross damage in the small intestine (Tanaka et al., 2002b). We further demonstrated that L-NAME given together with rofecoxib did produce intestinal damage, although inhibition of NO production by L-NAME alone induced no damage, the finding being similar to that observed under inhibition of both COX-1 and COX-2 (Tanaka et al., 2002b). Furthermore, we found that L-NAME up-regulated the expression of COX-2 mRNA in the intestine, similar to SC-560, a key to the occurrence of intestinal damage associated with COX-2 inhibition.

Wallace et al. (2000) recently reported that neither SC-560 nor celecoxib induced gastric lesions, but their combination caused damage in the stomach, suggesting that inhibition of both COX-1 and COX-2 is required for the occurrence of NSAID-induced gastric injury. We also showed that these two agents when administered together produced damage in the intestine, although neither of these agents alone induced any damage (Tanaka et al., 2002b). The present findings together with those data indicate that COX-2 as well as COX-1 plays a role in maintaining the mucosal integrity of the gastrointestinal tract and that the adverse reaction of NSAID is not accounted for solely by inhibition of COX-1.

Interestingly, we found that inhibition of NO production by L-NAME, the nonselective NOS inhibitor, also provoked intestinal damage in the presence of rofecoxib, in an L-arginine-sensitive manner. This effect was not, however, mimicked by aminoguanidine, the selective iNOS inhibitor, given together with rofecoxib. It was also found that the administration of L-NAME, but not aminoguanidine, up-regulated COX-2 expression in the small intestine, similar to SC-560. We have previously reported that the PGE2 content of the small intestine was markedly decreased by SC-560, yet gradually recovered from 6 h after the administration, in a rofecoxib-sensitive manner (Tanaka et al., 2002b). We confirmed in the present study that the PGE2 content in SC-560-treated animals was recovered 12 h later to the levels equivalent to those in normal rats, although it was markedly decreased at 3 h after the administration of SC-560. Interestingly, L-NAME had no effect on mucosal PGE2 production when determined 3 h after administration, but the PGE2 content in these rats increased 12 h later, in a COX-2-dependent manner. Certainly, L-NAME at the dose used decreases NO production in the small intestine (Tanaka et al., 1999). Since NO produced by NOS plays a role in the regulation of various functions of the small intestine, mimicking the role of COX-1/PGs (Tanaka et al., 2001), it is assumed that a rapid up-regulation of COX-2 expression might represent a redundant or compensatory response to inhibition of PG or NO biosynthesis and contribute to the maintenance of mucosal integrity under such adverse conditions. Because L-NAME causes a persistent increase in systemic blood pressure, it is possible that such blood pressure changes might account partly for the intestinal ulcerogenic action of this agent in the presence of rofecoxib; however, in a preliminary study, we did not observe any damage in the intestinal mucosa following administration of norepinephrine plus rofecoxib, arguing against the above possibility (unpublished data).

We previously reported that endogenous NO exerts a dual action in the pathogenesis of indomethacin-induced intestinal lesions; NO generated by NOS expressed constitutively, cNOS, is protective against indomethacin, whereas NO derived by iNOS plays a key pathogenetic role in the ulcerogenic process (Tanaka et al., 1999). Laszlo et al. (1994) demonstrated that following indomethacin administration, the early inhibition of NO synthase by L-NAME leads to acute microvascular injury in the rat intestine, indicating a protective role of NO formed by cNOS in the intestine. Aminoguanidine is known to prevent the intestinal ulceration induced by indomethacin (Tanaka et al., 1999; Takeuchi et al., 2002). In the present study, this agent also prevented the intestinal damage induced by L-NAME plus rofecoxib, suggesting the involvement of iNOS/NO in the pathogenesis of these lesions.

At present, the exact mechanism by which COX-2 expression is up-regulated following inhibition of L-NAME or SC-560 remains unknown. Because the expression of COX-2 induced by L-NAME was mitigated by pretreatment with L-arginine, it is likely that the process is associated with inhibition of NO production. Interestingly, the up-regulation of COX-2 expression and PGE2 production was also attenuated by atropine at the dose that inhibited intestinal hypermotility in response to L-NAME, suggesting a relationship between the hypermotility and COX-2 expression. Since inhibition of intestinal hypermotility resulted in suppression of bacterial invasion in the mucosa (Takeuchi et al., 2002), and enterobacteria induce iNOS expression through release of endotoxin [lipopolysaccharide (LPS)] (Boughton-Smith et al., 1993), it is possible that COX-2 expression is up-regulated by LPS, similar to iNOS expression. In a preliminary study, we observed that the expression of COX-2 following SC-560 treatment was attenuated by ampicillin due to sterilizing action, suggesting a close relationship between COX-2 expression and enterobacteria (Tanaka et al., 2004). As shown in the present study, both L-NAME and SC-560 enhanced bacterial invasion in the intestinal mucosa. Thus, it is possible that COX-2 expression is up-regulated by LPS under inhibition of the production of NO or PGs. These results strongly support the idea that the expression of COX-2 induced by L-NAME is associated with hypermotility and bacterial invasion. This idea was further supported by the fact that the intestinal ulcerogenic response to L-NAME plus rofecoxib was also significantly prevented by prior administration of ampicillin as well as aminoguanidine.

It is believed that NO produced by cNOS contributes to maintaining the mucosal integrity of the gastrointestinal tract by modulating various functions, such as mucosal blood flow and mucus secretion (Whittle, 1994). The mucus layer acts as a barrier to bacterial invasion in the mucosa, the major pathogenetic event in NSAID-induced intestinal damage (Tanaka et al., 2001, 2002c; Kunikata et al., 2002). We observed in the present study that intestinal mucus secretion was decreased by L-NAME as well as SC-560, suggesting a role for NO in the secretion, similar to PGs. It is assumed that endogenous NO as well as PGs hamper the enterobacterial invasion in the mucosa by stimulating the secretion of mucus and by increasing the mucus gel thickness. On the other hand, we previously reported an increase of intestinal motility following administration of NSAID and suggested the involvement of a hypermotility response in the pathogenesis of NSAID-induced intestinal damage (Kunikata et al., 2002; Takeuchi et al., 2002; Tanaka et al., 2002c). In the present study, we observed that intestinal motility was increased by both SC-560 and L-NAME but not aminoguanidine, again with a relation between this response and inhibition of COX-1 and cNOS. Abnormal hypermotility caused
by NSAID may disrupt the mucus layer over the epithelium, promoting enterobacterial invasion.

In conclusion, the present study suggests that intestinal damage occurs by the inhibition of both cNOS and COX-2 as well as both COX-1 and COX-2. The inhibition of NO production by l-NAME caused an increase in intestinal motility and a decrease in intestinal mucus secretion, resulting in enhancement of bacterial invasion, the events similar to those observed under conditions of PG deficiency caused by SC-560 through COX-1 inhibition; however, inhibition of cNOS or COX-1 up-regulates the expression of COX-2 through intestinal hypermotility, and this may counteract the deleterious events caused by endogenous NO or PG deficiency. Although cNOS exists in two isoforms, endothelial NOS and neuronal NOS, it remains unknown at present which type of cNOS contributes to the integrity of small intestinal mucosa. Finally, the present findings strongly suggest a role for COX-2 in maintaining the mucosal integrity of the small intestine.

References


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