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Factors Involved in Upregulation of Inducible Nitric Oxide Synthase in Rat Small Intestine Following Administration of Nonsteroidal Anti-inflammatory Drugs

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We investigated the functional mechanisms underlying the expression of inducible nitric oxide (NO) synthase (iNOS) in the rat small intestine following the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) and found a correlation with the intestinal ulcerogenic properties of NSAIDs. Conventional NSAIDs (indomethacin, dicrofenac, naproxen, and flurbiprophen), a selective cyclooxygenase (COX)-1 inhibitor (SC-560) and a selective COX-2 inhibitor (rofecoxib) were administered p.o., and the intestinal mucosa was examined 24 hours later. Indomethacin decreased prostaglandin E_2 (PGE_2) production in the intestinal mucosa and caused intestinal hypermotility and bacterial invasion as well as the upregulation of iNOS expression and NO production, resulting in hemorrhagic lesions. Other NSAIDs similarly inhibited PGE_2 production and caused hemorrhagic lesions with intestinal hypermotility as well as iNOS expression. Hypermotility in response to indomethacin was prevented by both PGE_2 and atropine but not ampicillin, yet all these agents inhibited not only bacterial invasion but also expression of iNOS as well, resulting in prevention of intestinal lesions. SC-560, but not rofecoxib, caused a decrease in PGE_2 production, intestinal hypermotility, bacterial invasion, and iNOS expression, yet this agent neither increased iNOS activity nor provoked intestinal damage because of the recovery of PGE_2 production owing to COX-2 expression. Food deprivation totally attenuated both iNOS expression and lesion formation in response to indomethacin. In conclusion, the expression of iNOS in the small intestine following administration of NSAIDs results from COX-1 inhibition and is functionally associated with intestinal hypermotility and bacterial invasion. This process plays a major pathogenic role in the intestinal ulcerogenic response to NSAIDs.

KEY WORDS: NSAID; intestinal damage; iNOS expression; COX-1 inhibition; intestinal motility; enterobacteria.

Nonsteroidal anti-inflammatory drugs (NSAIDs) cause intestinal damage as an adverse reaction in experimental animals and in humans (1–3). A number of factors such as bacterial flora, neutrophils, and inducible nitric oxide (NO) synthase (iNOS) are involved in the pathogenesis of these lesions (4, 5), yet a deficiency of endogenous prostaglandins (PGs) owing to inhibition of cyclooxygenase (COX) is of prime importance in the background for the ulcerogenic response to NSAIDs (6). Recently, several groups including our own (7–11) reported that the gastrointestinal ulcerogenic properties of NSAIDs are not solely explained by the inhibition of COX-1 and require the inhibition of both COX-1 and COX-2. Furthermore,

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we reported the upregulation of COX-2 expression in these tissues following administration of NSAIDs and demonstrated that the inhibition of COX-2 is a key to NSAID-induced gastrointestinal damage (10, 11).

NSAIDs, however, do not induce damage in the small intestine of germ-free animals or even fasting rats (1), suggesting that the presence of enterobacteria is essential for the development of intestinal lesions. Because enterobacteria release endotoxin (lipopolysaccharide), which causes the upregulation of iNOS expression and overproduction of NO in the gut (12), it is believed that NO plays a pathogenic role in the intestinal ulcerogenic response induced by NSAIDs. Indeed, several studies reported that indomethacin-induced intestinal lesions were prevented by both dexamethasone, an inhibitor of iNOS expression, and aminoguanidine, a relatively selective iNOS inhibitor (13, 14). However, functional mechanisms underlying the upregulation of iNOS expression or the relationship of COX inhibition to this event remain to be explored.

In the present study, we examined whether other conventional NSAIDs as well as a selective COX-1 or COX-2 inhibitor upregulate the expression of iNOS in the rat small intestine, similar to indomethacin, and investigated the functional mechanisms responsible for the upregulation by evaluating the influences of various drugs that prevent indomethacin-induced intestinal lesions and other functional changes (13–15).

MATERIALS AND METHODS

Animals. Male Sprague Dawley rats (weighing 220–260 g; Nippon Charles River, Shizuoka, Japan) were used. Studies were carried out using 5–6 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 Ulcerogenic Properties. The animals were administered orally with an NSAID (nonselective COX inhibitor) such as indomethacin (10 mg/kg), diclofenac (40 mg/kg), flurbiprofen (20 mg/kg), naproxen (40 mg/kg), SC-560 (a selective COX-1 inhibitor, 10 mg/kg) (16), or rofecoxib (a selective COX-2 inhibitor, 10 mg/kg) (17), and humanely killed 24 hours later. 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 ($\times 10$). The area (mm^2) of macroscopically visible lesions was measured, summed per small intestine, and used as a lesion score. The examiner measuring the lesions was blind to treatment given to the animals. In some cases, the effect of 16,16-dimethyl PGE_2 ($dmPGE_2$; 30 μg/kg), atropine (3 mg/kg), aminoguanidine (20 mg/kg), or ampicillin (800 mg/kg) on the intestinal lesions was examined. $DMPGE_2$, atropine, or aminoguanidine was given s.c. twice, 30 minutes before and 9 hours after indomethacin; ampicillin was given p.o. twice, 24 hours and 30 minutes before. The doses of these agents were selected to provide significant protection against indomethacin-induced intestinal damage, according to our previous papers (13–15). In some cases, the animals were deprived of food for 18 hours before the administration of indomethacin.

Determination of Mucosal PGE_2 Content. PGE_2 levels in the small intestinal mucosa were measured after the p.o. administration of various COX inhibitors, including indomethacin (10 mg/kg), diclofenac (40 mg/kg), flurbiprofen (20 mg/kg), naproxen (40 mg/kg), SC-560 (10 mg/kg), and rofecoxib (10 mg/kg). The animals were humanely killed under deep ether anesthesia 3 hours after the administration, and the small intestinal tissue was isolated, weighed, and placed in a tube containing 100% ethanol plus 0.1 mol indomethacin (18). Then, the tissues were homogenized using a polytron homogenizer (IKA, Tokyo, Japan) and centrifuged at 10,000 rpm for 10 minutes at 4°C. After the supernatant of each sample had been evaporated with N_2 gas, the residue was resolved in assay buffer and used for the determination of PGE_2. The concentration of PGE_2 was measured using a PGE_2 enzyme immunoassay kit (Amersham Pharmacia Biotech, UK).

Analysis of nNOS and iNOS mRNA Expression. Animals were humanely killed under deep ether anesthesia 6 hours after the p.o. administration of various COX inhibitors, including indomethacin (10 mg/kg), diclofenac (40 mg/kg), flurbiprofen (20 mg/kg), naproxen (40 mg/kg), SC-560 (10 mg/kg), and rofecoxib (10 mg/kg), and their small intestines were removed, frozen in liquid nitrogen, and stored at 80°C prior to use. In some rats, $dmPGE_2$ (30 μg/kg) or atropine (3 mg/kg) was given s.c. 1 hour before indomethacin or SC-560; ampicillin was given p.o. twice, 24 hours and 30 minutes before. In addition, some rats were deprived of food for 18 hours before the administration of indomethacin. Intestinal tissue samples were pooled from 2–3 rats for extraction of total RNA, which was prepared using a single-step acid phenol-chloroform extraction procedure with Trizole (Gibco, Gaithersburg, Md, USA). Total RNA primed by random hexadeoxy ribonucleotide was reverse transcribed with the Superscript pre-amplification system (Gibco). The sequences of sense and antisense primers for rat nNOS and iNOS are referred to in a previous paper (19) (Table 1). For rat glyceraldehyde-3-phosphate dehydrogenase

TABLE 1. SEQUENCES OF SENSE AND ANTISENSE PRIMERS FOR RAT nNOS AND iNOS

Enzyme	*Sequences*	*PCR Product*
nNOS		602 bp
Sense	5′-GAATACCAGCCTGATCCATGGAA-3	
Antisense	5′-TCCTCCAGGAGGGTGTCCACCGCATG-3′	
iNOS		651 bp
Sense	5′-CGGTTCACAGTCTTGGTGAAAG-3′	
Antisense	5′-CAGGTGTTCCCCAGGTAGGTAG-3′	

(G3PDH) gene, a constitutively expressed gene, the sequence was 5′-GAACGGGAAGCTCACTGGCATGGC-3′ for the sense primer and 5′-TGAGGTCCACCACCCTGTTGCTG-3′ for the antisense primer, giving rise to 310 bp (20). An aliquot of the Reverse Transcription (RT) reaction product served as a template in 30 cycles with 0.5 minutes of denaturation at 95°C and 1 minute of extension at 68°C using the Advantage 2 polymerase mixture (Clontech, Mountain View, Calif, USA) on a thermal cycler (Takara TP-240). A portion of the PCR mixture was electrophoresed in 1.8 % agarose gel in TAE buffer (40 mmol Tris, 2 mmol EDTA, and 20 mmol acetic acid, pH 8.1), and the gel was stained with ethidium bromide and photographed. The PCR product was subcloned into pGEM-T easy vector (Promega, Madison, Wisc, USA), and the sequence was determined by the dideoxy chain termination method with a Big Dye terminator v3.0 Cycle Sequencing kit (Biosystems, Middletown, Conn, USA), using an automatic DNA sequencer (ABI Prism 310 Genetic Analyser; Biosystems). The sequence of the PCR product was analyzed using the BLAST program (NBCI).

Determination of Small Intestinal Motility. Intestinal motility was determined using a miniature balloon according to previous papers (15, 21). In brief, the rats were 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 minutes, intestinal motility was monitored on a recorder (U-228, Tokai-Irika, Tokyo, Japan) as intraluminal pressure changes, through a pressure transducer and polygraph device (Nihon Kodan, Ibaragi, Japan). Indomethacin (10 mg/kg), SC-560 (10 mg/kg), or rofecoxib (10 mg/kg) was given intraduodenally after basal intestinal motility had stabilized, and the motility was measured for 3 hours thereafter. In some cases, dmPGE$_2$ (30 μg/kg) or atropine (3 mg/kg) was given s.c. 2 hours after the administration of indomethacin; ampicillin (800 mg/kg) was given intraduodenally 2 hours before indomethacin. Quantitation of intestinal motility was made by measuring the area of motility changes on a recording sheet using National Institutes of Health (NIH) image 1.61 (NIH, Bethesda, MD, USA). Data are expressed as the motility index (arbitrary units).

Determination of NOS Activity. The animals were humanely killed under deep ether anesthesia 24 hours after the administration of indomethacin (10 mg/kg, s.c.), SC-560 (10 mg/kg), or rofecoxib (10 mg/kg), and their small intestines were removed. After rinsing the intestine with cold saline, the mucosa was scraped with glass slides, weighed, and used for the determination of NOS activity. In some cases, dmPGE$_2$ or atropine was given s.c. twice 30 minutes before and 9 hours after indomethacin; ampicillin was given p.o. twice 24 hours and 30 minutes before. NOS activity was measured by determining the conversion of radiolabeled L-arginine to citrulline, according to the method described by Brown *et al.* (22). The tissue was homogenized in ice-cold buffer (Tris-HCl 50 mmol, sucrose 32 mmol, dithiothreitol 1 mmol, leupeptin 10 μg/mL, and aprotinin 2 μg/mL), adjusted to pH 7.4 with NaOH, and centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was incubated for 60 minutes at 37°C in a reaction buffer containing (^{3}H)-L-arginine at 0.5 μCi/mL. The level of activity of constitutive NOS (cNOS) was determined from the difference in the presence and absence of 1 mmol EGTA; the activity of iNOS was evaluated in the presence of 1 mmol EGTA. Protein content was estimated using the spectrophotometric assay as described above, and the NOS activity was expressed as pmol/min/mg protein.

Determination of NO Levels. Because aminoguanidine is an inhibitor of iNOS activity, we measured the amount of NO generated in the intestinal mucosa indirectly as nitrite/nitrate (NO_2^- and NO_3^-), in the absence or presence of aminoguanidine. Under deep ether anesthesia, the animals were humanely killed 24 hours after administration of indomethacin (10 mg/kg, s.c.), and their small intestines were removed. Aminoguanidine (20 mg/kg, s.c.) was given twice, at 30 minutes and 6 hours after indomethacin. After the rinsing of the intestine with cold saline, the mucosa (a 10-cm section starting 30 cm proximal to the ileocecal junction) was scraped with glass slides and homogenized in 50 mmol $KHPO_4$ buffer, before being centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant NO levels were measured by the Griess reaction-dependent method, described by Green *et al.* (23), after reduction of NO_3^- to NO_2^- with nitrate reductase. Nitrites were incubated with Griess reagent (0.1% naphthylene diamine dihydrochloride and 1% sulfanilamide in 2.5% H_3PO_4) for 10 minutes at room temperature, and the absorbance at 545 nm was measured. For the standard curve, sodium nitrite was used.

Determination of Enterobacterial Counts. The enterobacteria were enumerated according to a modified method originally described by Reuter *et al.* (24). At 3, 6, 12, and 24 hours after the administration of indomethacin (10 mg/kg, s.c.), SC-560 (10 mg/kg), or rofecoxib (10 mg/kg, p.o.), the animals were humanely killed under deep ether anesthesia and their small intestines were removed. DMPGE$_2$, atropine, or aminoguanidine was given subcutaneously twice—30 minutes before and 9 hours after indomethacin; ampicillin was given orally twice—24 hours and 30 minutes before. In some cases, animals were deprived of food for 18 hours before the administration of indomethacin, and the enterobacterial count was measured 6 hours after indomethacin treatment. After the intestine had been rinsed with sterile saline, the mucosa was scraped with glass slides, weighed, and homogenized in 1 mL of sterile phosphate-buffered saline per 100 mg of wet tissue. Aliquots of the homogenate were placed on blood agar and GAM agar (Nissui, Osaka, Japan). Blood agar plates were incubated at 37°C for 24 hours under aerobic conditions, and GAM agar plates were incubated for 48 hours under standard anaerobic conditions (BBL Gas Pack Pouch Anaerobic System, Becton Dickinson, Mountain View, Calif, USA). Plates containing 10–200 colony-forming units (CFU) were examined for numbers of enterobacteria, and the data were expressed as log CFU/g tissue.

Preparation of Drugs. The drugs used were indomethacin (Sigma Chemicals, St. Louis, Mo, USA), SC-560 (Cayman Chemical, Ann Arbor, Mich, USA), rofecoxib (synthesized by our group), ampicillin, atropine, aminoguanidine (Nakarai Tesque, Kyoto, Japan), 16,16-dimethyl prostaglandin E$_2$ (dmPGE$_2$; Funakoshi, Tokyo, Japan), and urethane (Tokyo Kasei, Tokyo, Japan). All COX inhibitors were suspended in a hydroxypropyl cellulose solution (Wako, Osaka, Japan). DMPGE$_2$ was first dissolved in absolute ethanol and then diluted with saline to a desired concentration. Other agents were dissolved in saline. Each agent was prepared immediately before

use and given i.p., p.o., i.d., or s.c. in a volume of 0.5 mL per 100 g body weight.

Statistics. Data are presented as the means ± SE of 4–8 rats per group. Statistical analyses were performed using the -tailed Dunnett's multiple comparison test, and values of $P < .05$ were considered significant.

RESULTS

Effects of Various NSAIDs on the Intestinal Mucosa, PGE_2 Levels and iNOS Expression

Oral administration of conventional NSAIDs produced hemorrhagic damage in the small intestinal mucosa within 24 hours; the lesion score being 215.6 ± 15.2 mm^2, 148.3 ± 14.7 mm^2, 217.1 ± 22.4 mm^2, and 181.3 ± 24.6 mm^2, respectively, for indomethacin (10 mg/kg), diclofenac (40 mg/kg), flurbiprofen (20 mg/kg), and naproxen (40 mg/kg) (Figure 1). The generation of PGE_2 in the small intestine was significantly inhibited by these agents, and the mucosal PGE_2 level in all cases had decreased by 90% when determined 3 hours after the administration.

As demonstrated previously (14), iNOS mRNA expression was upregulated at 6 hours after the administration of indomethacin, although the expression was not detected in the small intestine of vehicle-treated rats (Figure 2A).

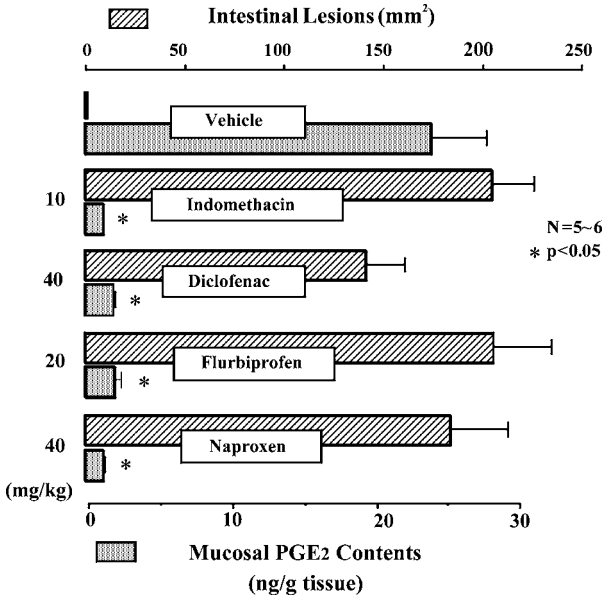

Fig 1. Intestinal ulcerogenic response of various NSAIDs and the effect of these agents on the mucosal PGE_2 level in the rat small intestine. The animals were administered indomethacin (IND: 10 mg/kg), dicrofenac (DIC: 40 mg/kg), flurbiprofen (FLU: 20 mg/kg), or naproxen (NAP: 40 mg/kg) p.o., and humanely killed 3 or 24 hours later for the evaluation of PGE_2 or lesions, respectively. Data are presented as the means ± SEM for 5–6 rats. *Significant difference from vehicle, at $P < .05$.

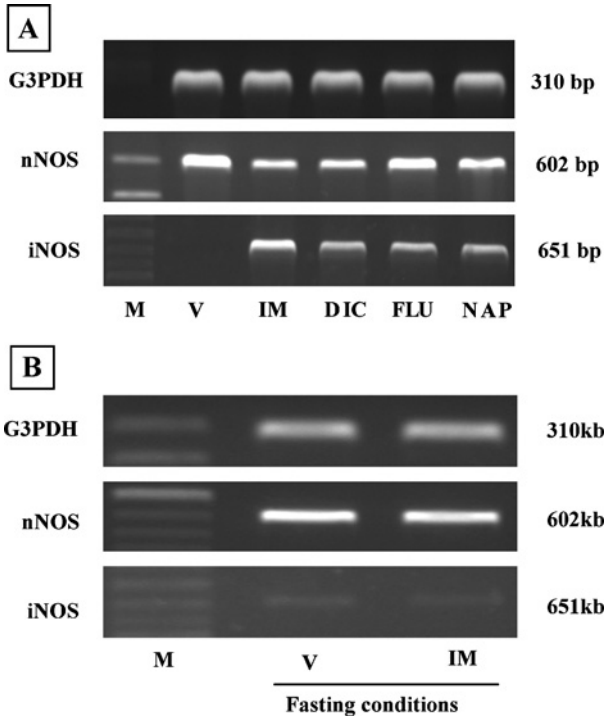

Fig 2. Gene expression of nNOS and iNOS in the intestinal mucosa following administration of various NSAIDs in normal fed rats (A) and the effect of food deprivation on the expression of iNOS mRNA induced by indomethacin (B). (A) Animals were administered indomethacin (IND: 10 mg/kg), dicrofenac (DIC: 40 mg/kg), flurbiprofen (FLU: 20 mg/kg), or naproxen (NAP: 40 mg/kg) p.o., and humanely killed 6 hours later. (B) Animals with or without 18 hours fasting were given indomethacin (10 mg/kg) s.c. and humanely killed 6 hours later. M, marker; V, vehicle.

Other NSAIDs tested also upregulated iNOS expression, similar to indomethacin. The expression of nNOS mRNA was observed in vehicle-treated rats and remained unchanged after the administration of NSAIDs. On the other hand, the upregulation of iNOS mRNA expression in the small intestine was not observed in rats fasted for 18 hours before the indomethacin treatment (Figure 2B).

Intestinal Lesions and Various Changes Induced by Indomethacin Time-Course Changes

A single p.o. administration of indomethacin (10 mg/kg) decreased mucosal PGE_2 levels to <10% of basal values at 3 hours after dosing, and this effect persisted for 24 hours (data not shown). The intestinal lesions induced by indomethacin occurred in a time-dependent manner; a few mild lesions were observed at 6 hours and severe hemorrhagic lesions at 12 and 24 hours (Figure 3). The iNOS activity observed in the intestinal mucosa of normal rats was minimal but increased significantly from 6 hours following administration of indomethacin, further strengthening to 2.3 ± 0.5 pmol/min/mg protein

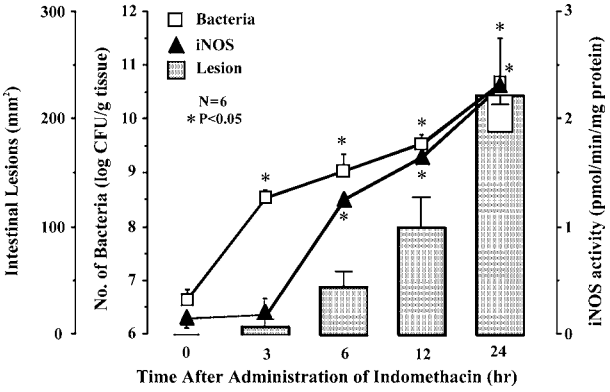

Fig 3. Time-course of changes in lesion formation, iNOS activity, and the enterobacterial number (aerobic) in the rat intestinal mucosa after administration of indomethacin. Animals were given indomethacin (10 mg/kg) p.o. and humanely killed 3, 6, 12, or 24 hours later. Data are presented as the means ± SEM for 5–6 rats.

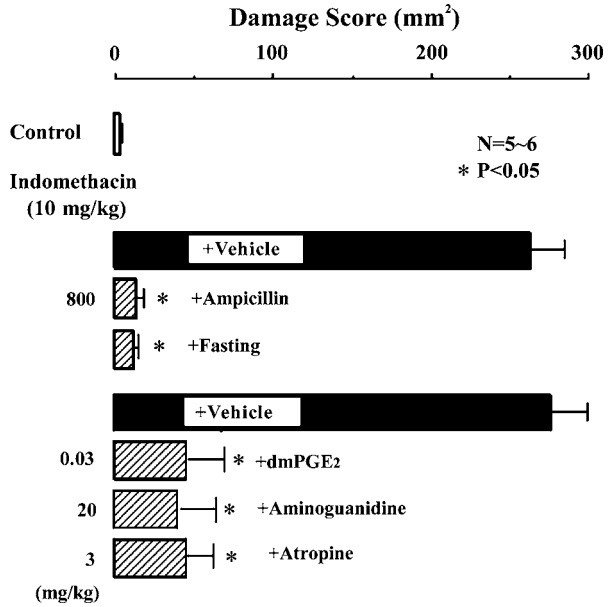

Fig 4. Effects of various agents and food deprivation on indomethacin-induced intestinal lesions in rats. Animals were given indomethacin p.o. at a dose of 10 mg/kg and humanely killed 24 hours later. $DMPGE_2$ (30 μg/kg), atropine (3 mg/kg), and aminoguanidine (20 mg/kg) were given s.c. twice −30 minutes before and 9 hours after indomethacin; ampicillin (800 mg/kg) was given p.o. twice −24 hours and 30 minutes before indomethacin. Food was withheld for 18 hours before the indomethacin treatment. Data are presented as means ± SEM from 5–6 rats. *Significant difference from vehicle, at $P < .05$.

at 24 hours. On the other hand, the number of aerobic bacteria in the intestinal mucosa had already increased at 3 hours after administration of indomethacin, from 6.63 ± 0.20 log CFU/g tissue to 8.88 ± 0.15 log CFU/g tissue, the values increasing further with time and reaching 10.94 ± 0.31 log CFU/g tissue at 24 hours. As evidenced from these data, the bacterial invasion apparently preceded the increase in iNOS activity as well as the development of damage in the small intestine.

Effects of Various Agents on Indomethacin-Induced Intestinal Responses

Lesions. The development of indomethacin-induced intestinal lesions was significantly prevented by ampicillin (800 mg/kg, p.o.), the degree of inhibition being 88.3% (Figure 4). Treatment of the animals with $dmPGE_2$ (30 μg/kg, s.c.), atropine (3 mg/kg, s.c.), or aminoguanidine (20 mg/kg, s.c.) also significantly reduced the severity of these lesions. In addition, the development of intestinal lesions was almost totally prevented by food deprivation for 18 hours before indomethacin treatment, the inhibition being 90.2%.

Intestinal Motility. Intestinal motility was enhanced following intraduodenal administration of indomethacin (10 mg/kg), in regard to both the amplitude and frequency of contraction (Figure 5). The enhanced intestinal motility caused by indomethacin was significantly inhibited by subsequent administration of $dmPGE_2$ (30 μg/kg) or atropine (3 mg/kg), resulting in fluctuations at the baseline that were difficult to characterize as contraction spikes. However, ampicillin (800 mg/kg) had no effect on the enhanced intestinal motility seen after administration of indomethacin.

Enterobacterial Number. Twenty-four hours after the administration of indomethacin (10 mg/kg, s.c.), the enterobacterial numbers in both aerobic and anaerobic conditions were about 1000-fold greater than the control levels, the values being 10.61 ± 0.37 log CFU/g tissue and 10.58 ± 0.38 log CFU/g tissue, respectively (Figure 6). Ampicillin (800 mg/kg, p.o.) completely prevented bacterial invasion in indomethacin-treated animals, and the numbers of both aerobic and anaerobic bacteria decreased even below control levels seen in the normal mucosa. Likewise, $dmPGE_2$ (30 μg/kg, s.c.) and atropine (3 mg/kg, s.c.) suppressed the increase in bacterial invasion in the mucosa in response to indomethacin, and the values for both aerobic and anaerobic bacteria were significantly lower than those seen in the animals treated with indomethacin alone. Aminoguanidine had no effect on the bacterial invasion following indomethacin treatment. On the other hand, the increase in bacterial invasion was also totally prevented by food deprivation for 18 hours prior to indomethacin treatment (Table 2). Although the bacterial counts in both aerobic and anaerobic conditions increased significantly in the intestinal mucosa, being about 10-fold greater than the control levels, even at 6 hours after the administration

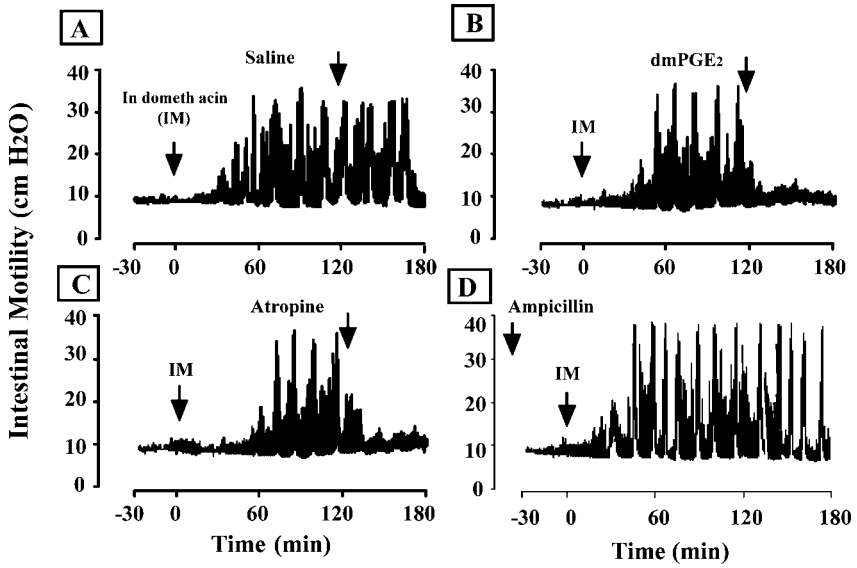

Fig 5. Representative recordings showing the effects of $dmPGE_2$ and atropine on the small intestinal motility enhanced by indomethacin in rats. Indomethacin (10 mg/kg) was given intraduodenally 1 hours after the basal motility had stabilized. Both $dmPGE_2$ (30 μg/kg) and atropine (3 mg/kg) were given s.c. 2 hours after the administration of indomethacin; ampicillin (800 mg/kg) was given intraduodenally 2 hours before indomethacin.

of indomethacin, such increases were not observed in the animals deprived of food.

iNOS Expression. Three hours after the administration of indomethacin, the expression of iNOS mRNA was observed in the intestinal mucosa. The upregulation of iNOS expression by indomethacin was inhibited by prior administration of either $dmPGE_2$ (30 μg/kg), ampicillin (800 mg/kg), or atropine (3 mg/kg) (Figure 7A). Neither of these agents had any effect on the expression of nNOS or of G3PDH in the intestinal mucosa.

iNOS Activity and NO Production. Indomethacin markedly increased the Ca^{2+}-independent NOS activity in the intestinal mucosa at 6 hours after the administration (Figure 7B) and increased the NO level in the intestinal mucosa from 160.8 ± 22.5 nmol/g tissue to 505.6 ± 43.5 nmol/g tissue at 24 hours posttreatment. The increase in iNOS activity following indomethacin treatment was significantly reduced by either $dmPGE_2$, ampicillin, or atropine, the inhibition being 75.3%, 91.4%, and 57.7%, respectively. Neither of these agents had any effect on cNOS activity in the intestinal mucosa (data not shown). Although aminoguanidine significantly decreased NO production in the mucosa, the level being 199.3 ± 42.8 nmol/g tissue, this agent had no effect on the production of NO in the normal mucosa.

Effects of Selective COX Inhibitors on iNOS Expression and Activity. It is known that the intestinal ulcerogenic property of NSAIDs is not accounted for solely by inhibition of COX-1 and requires inhibition of COX-2 as well, and that the inhibition of COX-1 upregulates the COX-2 expression, by which the deleterious influences of

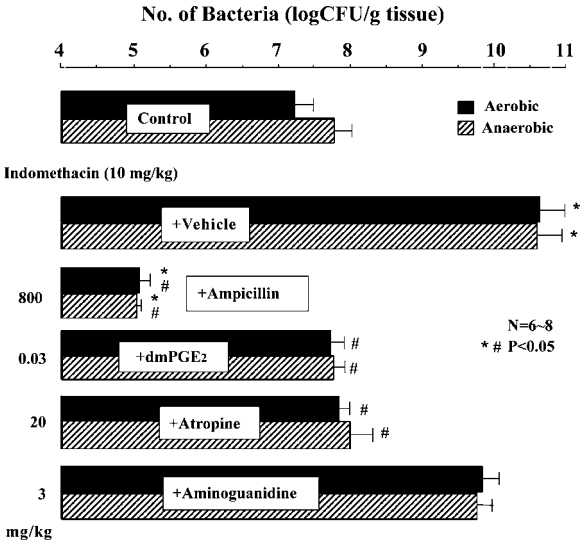

Fig 6. Effects of various drugs on bacterial counts in the rat small intestine. Animals were given indomethacin (10 mg/kg), and humanely killed 24 hours later. $dmPGE_2$ (30 μg/kg), atropine (3 mg/kg), and aminoguanidine (20 mg/kg) were given s.c. twice −30 minutes before and 9 hours after indomethacin; ampicillin (800 mg/kg) was given p.o. twice −24 hours and 30 minutes before indomethacin. All values are presented as the means ± SEM for 5–6 rats per group. Significant difference at $P < .05$: # from normal; *from vehicle.

TABLE 2. EFFECT OF INDOMETHACIN ON BACTERIAL COUNTS IN RAT SMALL INTESTINE UNDER NORMAL FEEDING OR FASTING CONDITIONS

Treatment	*No. of Rates*	*No. of Bacteria (log CFU/g tissue) Aerobic*	*Anaerobic*
Normal Feeding			
Control	5	6.54 ± 0.12	7.13 ± 0.15
Indomethacin	5	7.88 ± 0.44*	7.93 ± 0.31*
Fasting			
Control	5	5.79 ± 0.13	5.50 ± 0.39
Indomethacin	5	6.06 ± 0.18	5.77 ± 0.47

Note. All values are presented as the means ± SEM for 5 rats/group. Animals were given indomethacin (10 mg/kg), and humanely killed 6 hours later. Half the animals were deprived of food for 18 hours before the administration of indomethacin.
*Significant difference from control, at $P < .05$.

the prostaglandin the deficiency of PGs caused by COX-1 inhibition may be counteracted through additional PG production (11). So, we examined in this study whether or not the expression of iNOS is upregulated in the intestine following administration of the selective COX-1 or COX-2 inhibitor, together with their effects on intestinal motility, bacterial invasion, and iNOS activity.

As shown in Figure 8A, iNOS mRNA was expressed in the intestinal mucosa 6 hours after administration of SC-560 (10 mg/kg) but not rofecoxib (10 mg/kg), although it was not detected in the control rats given vehicle. However, neither SC-560 nor rofecoxib had any effect on Ca^{2+}-independent NOS activity, and the values were not significantly different from those observed in control rats (Figure 8B). Six hours after the administration of SC-560, the number of bacteria in both aerobic and anaerobic conditions was significantly increased to 8.25 ± 0.18 log CFU/g tissue and 8.07 ± 0.03 log CFU/g tissue, respectively (Figure 8C). By contrast, the bacterial count in the mucosa remained unchanged following administration of rofecoxib (10 mg/kg), and the values for both aerobic and anaerobic enterobacteria were not significantly different from those in the control group given vehicle alone. On the other hand, SC-560 enhanced intestinal motility, similar to indomethacin, and this effect persisted for over 3 hours; rofecoxib did not have any influence on intestinal motility and no clear contraction was observed before and after administration of this agent (Figure 8D).

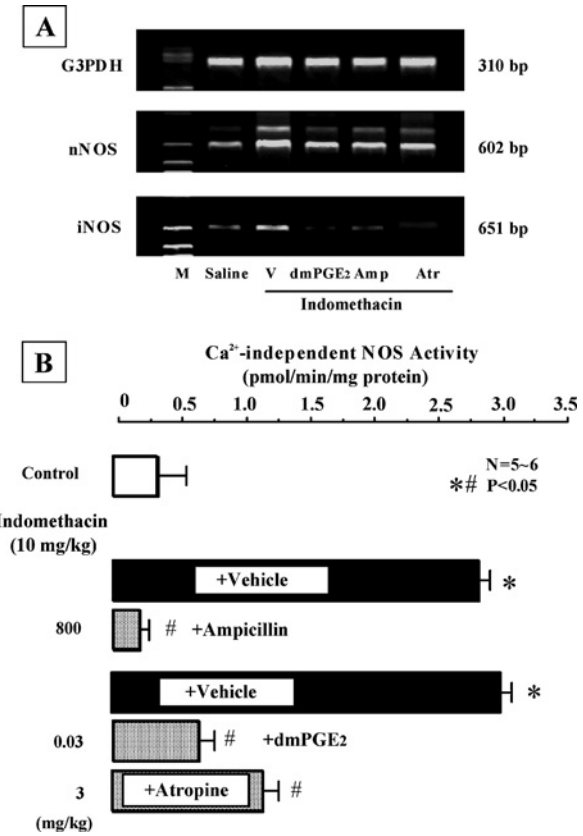

Fig 7. Effect of various drugs on the expression of iNOS mRNA (A) and Ca^{2+}-independent NOS activity (B) in the intestinal mucosa following indomethacin treatment. Animals were administered indomethacin (10 mg/kg) p.o., and the expression of iNOS or the iNOS activity was determined 6 hours or 24 hours later, respectively. $DMPGE_2$ (30 μg/kg) or atropine (3 mg/kg) was given s.c. twice −30 minutes before and 9 hours after indomethacin; ampicillin (800 mg/kg) was given p.o. twice −24 hours and 30 minutes before indomethacin. Data in B are presented as the means ± SEM for 5–6 rats. Significant difference at $P < .05$; *from control; # from vehicle.

DISCUSSION

The present study confirms the upregulation of iNOS expression in the rat small intestine following administration of NSAIDs and the role of iNOS/NO in the pathogenesis of NSAID-induced intestinal damage, and further demonstrated that this process was prevented by pretreatment with both atropine and ampicillin as well as the administration of $dmPGE_2$. We also observed that the selective COX-1 inhibitor SC-560 caused the upregulation of iNOS expression, together with intestinal hypermotility and bacterial invasion, suggesting the importance of PG deficiency owing to COX-1 inhibition as a background factor for this phenomenon. Because intestinal hypermotility is critical for bacterial invasion under conditions of PG deficiency caused by COX-1 inhibition (15), this functional alteration is a key to the iNOS expression after administration of NSAIDs and their intestinal ulcerogenic properties.

Consistent with our previous findings (14, 19), indomethacin upregulated the expression of iNOS mRNA and significantly increased Ca^{2+}-independent iNOS activity as well as NO production in the small intestine. The expression of iNOS was increased by other conventional NSAIDs such as dicrofenac, naproxen, and flurbiprofen

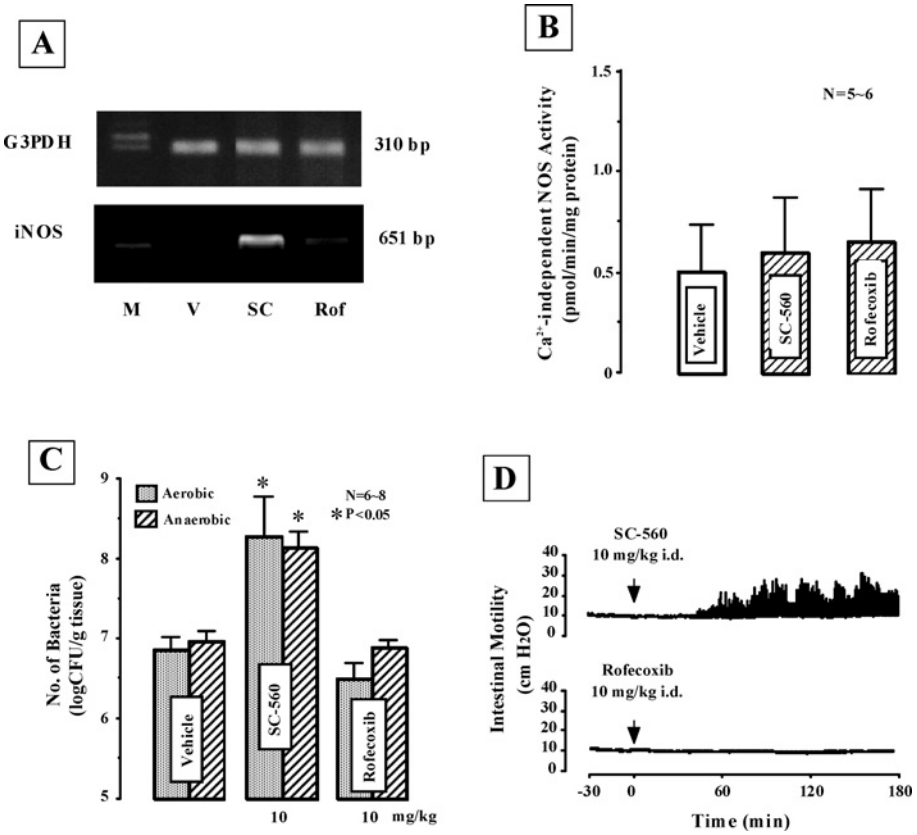

Fig 8. Gene expression of iNOS (A), iNOS activity (B), or enterobacterial count (C) in the intestinal mucosa, and intestinal motility (D) following administration of SC-560 or rofecoxib. (A–C) Animals were administered SC-560 (SC: 10 mg/kg) or rofecoxib (Rof: 10 mg/kg) p.o. (D) These agents were administered intraduodenally after basal intestinal motility had stabilized. Both the expression of iNOS mRNA and bacterial count were determined 6 hours later; the iNOS activity was measured 24 hours later. (A) M, marker; V, vehicle. (**B, C**) Data are presented as the means ± SE from 5–8 rats. *Significant difference from vehicle, at $P < .05$.

at doses that suppressed PG production and provoked intestinal lesions. These results suggest that the upregulation of iNOS expression is causally related to COX inhibition and plays a pathogenic role in NSAID-induced intestinal lesions. We found in the present study that the expression of iNOS was also upregulated in the intestinal mucosa following administration of SC-560, the selective COX-1 inhibitor, but not rofecoxib, the selective COX-2 inhibitor, indicating that this event is associated with inhibition of COX-1 activity.

It has been shown that indomethacin-induced intestinal lesions accompany various functional alterations, including intestinal hypermotility, bacterial invasion, and an increase of iNOS activity (14, 15). Interestingly, we found in the present study that the expression of iNOS induced by indomethacin was attenuated by atropine at a dose that inhibited the intestinal hypermotility in response to indomethacin. Because atropine by itself does not seem to have any influence on inflammatory changes, the observed effect may be brought about by the inhibition of intestinal hypermotility through anticholinergic action, suggesting a relationship between the hypermotility and iNOS expression. SC-560 also caused an increase in intestinal motility and the bacterial count in the mucosa, suggesting a role for COX-1 inhibition in intestinal hypermotility in response to NSAIDs as well as a causal relationship between these 2 events (19, 25). Because inhibition of the intestinal hypermotility resulted in suppression of bacterial invasion in the mucosa and because enterobacteria induce the expression of iNOS through the release of endotoxin (12), it is possible that intestinal hypermotility indirectly upregulates the expression of iNOS through bacterial invasion. These results strongly support the idea that the expression of iNOS following inhibition of COX-1 is closely associated with intestinal hypermotility and subsequent bacterial invasion. It is assumed that the abnormal hyper-

motility caused by NSAIDs disrupts the unstirred mucus layer over the epithelium, leading to the expedition of enterobacterial invasion. Certainly, ampicillin prevented the expression of iNOS seen after the indomethacin treatment, despite having no effect on the intestinal hypermotility response. Bertrand *et al.* (26) recently reported that the production of tumor necrosis factor-α (TNF-α) was linked to the toxicity of indomethacin in the small intestine, especially at the early stage of the ulcerogenic process. They also showed that TNF-α induces iNOS activity, which then acts as the effector pathway to produce injury. Thus, it is possible that TNF-α is also involved in the underlying mechanism of iNOS expression following administration of NSAIDs.

It should be noted that in this study SC-560 did not increase iNOS activity in the mucosa, despite upregulating iNOS mRNA expression. Because we observed both iNOS expression and NO production in the small intestine after the administration of indomethacin, there is no doubt that the upregulation of iNOS mRNA expression is followed by iNOS protein expression, resulting in production of NO in the small intestine. We previously reported that the mucosal PGE_2 level in the small intestine was markedly decreased by SC-560, yet recovered from 6 hours after the administration, in a rofecoxib-sensitive manner (11). This COX-2 expression may represent a compensatory response to the inhibition of PG biosynthesis caused by COX-1 inhibition, and the PGE_2 derived from COX-2 suppresses the deleterious events resulting from COX-1 inhibition (11, 25). Indeed, the severity of indomethacin-induced intestinal lesions was significantly reduced by later administration of 16,16-dimethyl PGE_2 as well as aminoguanidine, the selective iNOS inhibitor (11, 27). Thus, it is assumed that in the animals treated with SC-560, the PGE_2 produced by COX-2 inhibits iNOS activity, similar to aminoguanidine, despite upregulating iNOS expression. Alternatively, it is possible that the PGE_2 regulates the posttranscriptional regulatory mechanisms to decrease iNOS protein expression or increase the protein degradation.

At present, the source cells for iNOS expression in the intestinal mucosa remain unknown. We previously reported that neutrophils play a permissive role in NSAID-induced intestinal lesions, inasmuch as these lesions were significantly prevented by antineutrophil serum (13). These blood cells are a source of oxygen radicals and iNOS, and peroxynitrites formed by the interaction of NO with oxygen radicals may be detrimental in this model (28, 29). Lamarque *et al.* (30) also reported that iNOS activity in the duodenum and colon and expression of iNOS protein in the lamina propria inflammatory cells was increased in patients with ankylosing spondylitis compared to controls. In the present study, SC-560 by itself caused an increase of bacterial invasion and iNOS expression in the intestinal mucosa. However, as we previously reported, an increase of myeloperoxidase activity was observed only after the combined administration of SC-560 plus rofecoxib, not by SC-560 alone (19). Thus, cells other than neutrophils should be considered as the source of the iNOS expressed after the administration of SC-560. Indeed, we observed using immunohistochemistry that COX-2 expression occurred in intestinal epithelial cells after the administration of SC-560 or indomethacin (11). Because the expression of both iNOS and COX-2 is prevented by atropine and ampicillin as well as $dmPGE_2$ (25), intestinal epithelial cells could be the source cells of iNOS expression. Further study is needed to verify this point.

Given these findings, we conclude that the upregulation of iNOS expression in the intestinal mucosa following administration of indomethacin is functionally associated with intestinal hypermotility and enterobacterial invasion, both of which result from inhibition of COX-1. Therefore, intestinal hypermotility may be key to the expression of iNOS after the administration of NSAIDs and their intestinal ulcerogenic properties.

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REFERENCES

1. Robert A, Asano T: Resistance of germ-free rats to indomethacin-induced intestinal inflammation. Prostaglandins 14:333–341, 1987
2. Fang WF, Broughton A, Jacobson ED: Indomethacin induced intestinal inflammation. Am J Dig Dis 22:749–760, 1977
3. Bjarnason I, Zanelli G, Smith T, *et al.*: Nonsteroidal anti-inflammatory drug-induced intestinal inflammation in humans. Gastroenterology 93:480–489, 1987
4. Weissenborn U, Maedge S, Buettner D, Sewing KF: Indomethacin-increased gastrointestinal lesions in relation to tissue concentration, food intake and bacterial invasion in the rat. Pharmacology 30:32–39, 1985
5. Whittle BJR: Temporal relationship between cyclooxygenase inhibition, as measured by prostacyclin biosynthesis, and the gastrointestinal damage induced by indomethacin in the rat. Gastroenterology 80:94–98, 1981
6. Whittle BJR, Laszlo F, Evans AM, Moncada S: Inducible nitric oxide synthase and microvascular injury in the rat jejunum provoked by indomethacin. Br J Pharmacol 116:2286–2290, 1995
7. Wallace JL, McKnight W, Reuter BK, Vergnolle N: NSAID-induced gastric damage in rats: requirement for inhibition of both cyclooxygenase 1 and 2. Gastroenterology 119:706–714, 2000
8. Gretzer B, Maricic N, Respondek M, Schuligoi R, Peskar BM: Effects of specific inhibition of cyclo-oxygenase-1 and cyclo-

oxygenase-2 in the rat stomach with normal mucosa and after acid challenge. Br J Pharmacol 132:1565–1573, 2001
9. Takeuchi K, Tanaka A, Suzuki K, Mizoguchi H, Araki H: Gastrointestinal sparing anti-inflammatory drugs: effects on ulcerogenic and healing responses. Current Pharmaceutical Design 7:49–71, 2001
10. Tanaka A, Araki H, Hase S, Komoike Y, Takeuchi K: Inhibition of both COX-1 and COX-2 is required for development of gastric damage in response to nonsteroidal antiinflammatory drugs. J Physiol Paris 95:21–27, 2001
11. Tanaka A, Hase S, Miyazawa T, Takeuchi K: Up-regulation of COX-2 by inhibition of COX-1: A key to NSAID-Induced intestinal damage. J Pharmacol Exp Ther 300:754–761, 2002
12. Boughton-Smith N, Evans SM, Laszlo F, Whittle BJR, Moncada S: The induction of nitric oxide synthase and intestinal vascular permeability by endotoxin in the rat. Br J Pharmacol 110:1189–1195, 1993
13. Konaka A, Nishijima M, Tanaka A, Kunikata T, Kato S, Takeuchi K: Nitric oxide, superoxide radicals and mast cells in Pathogenesis of indomethacin-induced small intestinal lesions in rats. J Physiol Pharmacol 50:25–38, 1999
14. Tanaka A, Kunikata T, Konaka A, Kato S, Takeuchi K: Dual action of nitric oxide in pathogenesis of indomethacin-induced small intestinal ulceration in rats. J Physiol Pharmacol 50:405–417, 1999
15. Takeuchi K, Miyazawa T, Tanaka A, Kato S, Kunikata T: Pathogenic importance of intestinal hypermotility in NSAID-induced small intestinal damage in rats. Digestion 66:30–41, 2002
16. Smith CJ, Zhang Y, Koboldt CM, *et al*.: Pharmacological analysis of cyclooxygenase-1 in inflammation. Proc Natl Acad Sci USA 95:13313–13318, 1998
17. Chan CC, Boyce S, Bridear C, *et al*.: Pharmacology of a selective cyclooxygenase-2 inhibitor L-745,337: a novel non-steroidal anti-inflammatory agent with an ulcerogenic sparing effect in rat and non-human primate stomach. J Pharmacol Exp Ther 274:1531–1537, 1995
18. Futaki N, Takahashi S, Yokoyama M, Arai I, Higuchi S, Otomo S: NS-398, a new antiinflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclo-oxygenase (COX-2) activity in vitro. Prostaglandins 47:55–59, 1994
19. Tanaka A, Hase S, Miyazawa T, Ohno R, Takeuchi K: Role of COX-1 and COX-2 inhibition in NSAID-induced intestinal damage in rats: relation to various pathogenic events. J Pharmacol Exp Ther 303:1248–1254, 2002
20. Feng L, Sun W, Xia Y, *et al*.: Cloning two isoforms of rat cyclooxygenase: Differential regulation of their expression. Arch Biochem Biophys 307:361–368, 1993
21. Kunikata T, Umeda M, Tanaka A, Kato S, Takeuchi K: 16,16-dimetyl prostaglandin E_2 inhibits indomethacin-induced small intestinal lesions through EP3 and EP4 receptors. Dig Dis Sci 47:894–904, 2002
22. Brown JF, Tepperman BL, Hanson PJ, Whittle BJR, Moncada S: Differential distribution of nitric oxide synthase between cell fractions isolated from the rat gastric mucosa. Biochem Biophys Res Comm 184:680–685, 1992
23. Green LC, Tannebaum SR, Goldman P: Nitrite synthesis in the germ free and conventional rat. Science 212:56–58, 1981
24. Reuter BK, Davies NM, Wallace JL: Nonsteroidal anti-inflammatory drug enteropathy in rats: role of permeability, bacteria, and enterohepatic circulation. Gastroenterology 112:109–117, 1997
25. Tanaka A, Matsumoto M, Hayashi Y, Takeuchi K: Functional mechanism underlying cyclooxygenase-2 expression in rat small intestine following administration of indomethacin: relation to intestinal hypermotility. J Gastroenterol Hepatol 20:38–45, 2005
26. Bertrand V, Guimbaud R, Tulliez M, *et al*.: Increase in tumor necrosis factor-α production linked to the toxicity of indomethacin for the rat small intestine. Br J Pharmacol 124:1385–1394, 1998
27. Konaka A, Nishijima M, Tanaka A, Kato S, Takeuchi K: Roles of enterobacteria, nitric oxide and neutrophil in pathogenesis of indomethacin-induced small intestinal lesions in rats. Pharmacol Res 40:517–524, 1999
28. Evans SM, Whittle BJ: Interactive roles of superoxide and inducible nitric oxide synthase in rat intestinal injury provoked by non-steroidal anti-inflammatory drugs. Eur J Pharmacol 429:287–96, 2003
29. Mizoguchi H, Miyazawa T, Ogawa Y, Tanaka A, Kato S, Takeuchi K: Protective effect of rebamipide on indomethacin-induced intestinal damage in rats. J Gastroent Hepatol 16:1112–1119, 2001
30. Lamarque D, Nhieu JT, Breban M, *et al*.: Lymphocytic infiltration and expression of inducible nitric oxide synthase in human duodenal and colonic mucosa is a characteristic feature of ankylosing spondylitis. J Rheumatol 30:2428–2436, 2003