Role of Cyclooxygenase (COX)-1 and COX-2 Inhibition in Nonsteroidal Anti-Inflammatory Drug-Induced Intestinal Damage in Rats: Relation to Various Pathogenic Events

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ABSTRACT
We recently reported that cyclooxygenase (COX)-2 expression was up-regulated in the rat small intestine after administration of indomethacin, and this may be a key to nonsteroidal anti-inflammatory drug (NSAID)-induced intestinal damage. In the present study, we investigated the effect of inhibiting COX-1 or COX-2 on various intestinal events occurring in association with NSAID-induced intestinal damage. Rats without fasting were treated with indomethacin, SC-560 (a selective COX-1 inhibitor), rofecoxib (a selective COX-2 inhibitor), or SC-560 plus rofecoxib, and the following parameters were examined in the small intestine: the lesion score, the enterobacterial number, myeloperoxidase (MPO) and inducible nitric-oxide synthase (iNOS) activity, and intestinal motility. Indomethacin decreased mucosal prostaglandin (PG)E2 content and caused damage in the intestine within 24 h, accompanied by an increase in intestinal contractility, bacterial numbers, and MPO as well as iNOS activity, together with the up-regulation of COX-2 and iNOS mRNA expression. Neither SC-560 nor rofecoxib alone caused intestinal damage, but their combined administration produced lesions. SC-560, but not rofecoxib, caused intestinal hypermotility, bacterial invasion, and COX-2 as well as iNOS mRNA expression, yet the iNOS and MPO activity was increased only when rofecoxib was also administered. Although SC-560 inhibited the PG production, the level of PGE2 was restored 6 h later, in a rofecoxib-dependent manner. We conclude that inhibition of COX-1, despite causing intestinal hypermotility, bacterial invasion, and iNOS expression, up-regulates the expression of COX-2, and the PGE2 produced by COX-2 counteracts deleterious events, and maintains the mucosal integrity. This sequence of events explains why intestinal damage occurs only when both COX-1 and COX-2 are inhibited.

Nonsteroidal anti-inflammatory drugs (NSAIDs) cause intestinal damage as an adverse reaction in experimental animals and humans (Fang et al., 1977; Robert and Asano, 1977; Bjarnason et al., 1998). Although a number of elements such as bacterial flora, neutrophils, and inducible nitric-oxide synthase (iNOS) are involved in the pathogenesis of these lesions (Whittle, 1981; Weissenborn et al., 1985; Asako et al., 1992; Yamada et al., 1993; Konaka et al., 1999), a deficiency of endogenous prostaglandins (PGs) is of prime importance in the background for the ulcerogenic response to NSAIDs. This contention is supported by the fact that NSAID-induced gastric damage is prevented by supplementations of exogenous PGs (Kunikata et al., 2002a; Tanaka et al., 2002). We have recently reported the importance of intestinal hypermotility in the pathogenesis of NSAID-induced intestinal lesions (Kunikata et al., 2002b; Takeuchi et al., 2002). The deficiency of PGs caused by NSAIDs is due to an inhibition of both cyclooxygenase (COX)-1 and COX-2. Recently, several groups, including our own (Wallace et al., 2000; Gretzer et al., 2001; Tanaka et al., 2001, 2002), reported that the 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, suggesting a role for COX-2 as well as COX-1 in maintaining the integrity of the gastrointestinal mucosa. In addition, we demonstrated the up-regulation of COX-2 in these tissues after inhibition of COX-1 and suggested that this event is a key to NSAID-induced gastrointestinal damage (Tanaka et al., 2001, 2002). However, the pathogenic role of COX-1 or COX-2 inhibition in NSAID-induced intestinal damage remains unexplored.

In the present study, we examined the effect that inhibiting COX-1 or COX-2 has on the various functional changes.

ABBREVIATIONS. NSAID, nonsteroidal anti-inflammatory drug; iNOS, inducible nitric-oxide synthase; PG, prostaglandin; COX, cyclooxygenase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; OD, optical density; PCR, polymerase chain reaction; CFU, colony-forming units; NO, nitric oxide; SC-560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole.
observed in the small intestine in association with NSAID-induced intestinal damage in rats (Takeuchi et al., 2002), using SC-560, a selective COX-1 inhibitor, and rofecoxib, a selective COX-2 inhibitor, as well as indomethacin, a nonselective COX inhibitor, and investigated which event is most important to the ulcerogenic action of NSAIDs.

Materials and Methods

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

Evaluation of Small Intestinal Ulcerogenic Property. The animals were treated orally with 10 mg/kg indomethacin (a nonselective COX inhibitor), 10 mg/kg SC-560 (a selective COX-1 inhibitor) (Smith et al., 1998), 10 mg/kg rofecoxib (a selective COX-2 inhibitor) (Chan et al., 1995), or SC-560 plus rofecoxib, and killed 24 h 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 (10 × 10). The area (square millimeter) 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.

Determination of Mucosal PGE$_2$ Content. PGE$_2$ levels in the small intestinal mucosa were measured after p.o. administration of indomethacin (10 mg/kg), SC-560 (10 mg/kg), rofecoxib (10 mg/kg), or SC-560 plus rofecoxib. The animals were killed under deep ether anesthesia at various time points (3, 6, and 12 h) after the administration, and the small intestinal tissue was isolated, weighed, and processed. The mucosa was removed, frozen in liquid nitrogen, and stored at −80 °C. After the supernatant of each sample had been evaporated with N$_2$ gas, the residue was resolved in assay buffer and used for determination of PGE$_2$. The concentration of PGE$_2$ was measured using a PGE$_2$ enzyme immunoassay kit (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK).

Analysis of COX-1, COX-2, and iNOS mRNA Expression by Reverse Transcription-PCR. The animals were killed under deep ether anesthesia 6 h after p.o. administration of 10 mg/kg indomethacin, 10 mg/kg SC-560, or 10 mg/kg rofecoxib, and their small intestines were removed, frozen in liquid nitrogen, and stored at −80 °C until use. 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 hexadeoxynucleotide was reverse-transcribed with the SUPERSCRIPT preamplification system (Invitrogen). The sequences of sense and antisense primers for rat COX-1, COX-2, and iNOS are listed in Table 1 (Bredt et al., 1991; Lyons et al., 1992; Feng et al., 1993). For the rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a constitutively expressed gene, the sequence was 5′-GAACCGGAGACTCAGCTGGATGC-3′ for the sense primer and 5′-TGGAGTCCACACCCCTGTGTGCTG-3′ for the antisense primer, giving rise to a 310-base pair PCR product (Iso et al., 1995). An aliquot of the reverse-transcribed 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 TAE 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.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sequences</th>
<th>PCR Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1</td>
<td>Sense 5′-AACCGGAGACTTGGATGC-3′</td>
<td>887 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-TGGAGTCCACACCCCTGTGTGCTG-3′</td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>Sense 5′-TGGAGTCCACACCCCTGTGTGCTG-3′</td>
<td>702 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-TGGAGTCCACACCCCTGTGTGCTG-3′</td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>Sense 5′-AATGTTGAAGGTGTCCGGCAGC-3′</td>
<td>651 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-TGGAGTCCACACCCCTGTGTGCTG-3′</td>
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</tr>
</tbody>
</table>

| TABLE 1 | Sequences of sense and antisense primers for rat COX-1, COX-2 and iNOS |

Determination of Small Intestinal Motility. Intestinal motility was determined using a miniature balloon according to previous studies (Kunikata et al., 2002b; Takeuchi et al., 2002). In brief, the rat was anesthetized with urethane (1.25 g/kg i.p.), 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 device (Nihon Koden, Ibaragi, Japan). Indomethacin (10 mg/kg), SC-560 (10 mg/kg), or rofecoxib (10 mg/kg) was given i.d. after basal intestinal motility had well stabilized, and the motility was measured for 3 h thereafter. Quantitation of intestinal motility was done by measuring the area of motility changes in a recording sheet using NIH Image 1.61 (National Institutes of Health, Bethesda, MD), and the data are expressed as the motility index (arbitrary unit).

Determination of MPO and NOS Activities. The animals were killed under deep ether anesthesia 24 h after administration of indomethacin (10 mg/kg), SC-560 (10 mg/kg), rofecoxib (10 mg/kg), or SC-560 plus rofecoxib, 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 MPO and NOS activities.

MPO Activity. MPO activity was measured according to a modified version of the method of Bradley et al. (1982). The tissue was homogenized in 50 mM phosphate buffer containing 0.5% hexadecyltrimethyl-ammonium bromide, pH 6.0 (Sigma-Aldrich, St. Louis, MO), and centrifuged at 2000 rpm for 10 min at 4°C. The supernatant (100 μl) was added to 1.9 ml of 10 mM phosphate buffer, pH 6.0, and 1 ml of 1.5 M o-dianisidine hydrochloride (Sigma-Aldrich) containing 0.0005% (w/v) hydrogen peroxide. Then, the changes in absorbance at 450 nm were recorded on a Hitachi spectrophotometer (U-2000; Hitachi, Ibaraki, Japan), and the MPO activity was obtained from the slope of the reaction curve according to the following equation: specific activity (micromoles of H$_2$O$_2$ per minute per milligram of protein) = (OD/min)×(OD/mg)×(mg of protein).

NOS Activity. NOS activity was measured by determining the conversion of radiolabeled L-arginine to citrulline, according to the method described by Brown et al. (1992). The tissue was homogenized in ice-cold buffer (50 mM Tris-HCl, 32 mM sucrose, 1 mM dithiothreitol, 10 μg/ml aprotinin, and 2 μg/ml aprotonin), adjusted to pH 7.4 with NaOH, and centrifuged for 20 min at 10,000 rpm at 4°C. The supernatant (100 μl) was added to 1.9 ml of 10 mM phosphate buffer, pH 6.0, and 1 ml of 1.5 M o-dianisidine hydrochloride (Sigma-Aldrich) containing 0.0005% (w/v) hydrogen peroxide. Then, the changes in absorbance at 450 nm were recorded on a Hitachi spectrophotometer (U-2000; Hitachi, Ibaraki, Japan), and the MPO activity was obtained from the slope of the reaction curve according to the following equation: specific activity (micromoles of H$_2$O$_2$ per minute per milligram of protein) = (OD/min)×(OD/mg)×(mg of protein).
content was estimated by the spectrophotometric assay as described above, and the NOS activity was expressed as picomoles per minute per milligram of protein.

**Determination of the Number of Enterobacteria.** The enterobacteria were enumerated according to a modified method originally described by Reuter et al. (1997). Twenty-four hours after administration of 10 mg/kg indomethacin, 10 mg/kg SC-560, 10 mg/kg rofecoxib, or SC-560 plus rofecoxib, the animals were killed under deep ether anesthesia, and their small intestines were removed. After rinsing each intestine 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 h under aerobic conditions, whereas GAM 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 are expressed as log CFU/g tissue.

**Preparation of Drugs.** The drugs used were indomethacin (Sigma-Aldrich), SC-560 (Cayman Chemicals, Ann Arbor, MI), rofecoxib (synthesized in our laboratory), and urethane (Tokyo Kasei, Tokyo, Japan). All COX inhibitors were suspended in a hydroxy propyl cellulose solution (Wako Pure Chemicals, Osaka, Japan). The other drugs were dissolved in saline. All drugs were prepared immediately before use and administered p.o., i.d., or i.p. in a volume of 0.5 ml/100 g body weight.

**Statistics.** Data are presented as the mean ± S.E. of four to six 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 Various COX Inhibitors on Intestinal Mucosa and PGE₂ Content.** Oral administration of indomethacin, the nonselective COX inhibitor, damaged the small intestine within 24 h, the lesion score being 178.6 ± 21.2 mm² (Fig. 1). Neither the selective COX-1 inhibitor SC-560 (10 mg/kg) nor the selective COX-2 inhibitor rofecoxib (10 mg/kg) induced any gross damage in the small intestine during the same test period. However, when SC-560 was given together with rofecoxib, it did produce hemorrhagic lesions in the small intestine at an incidence of 100%, the lesion score being 95.6 ± 9.7 mm².

Indomethacin (10 mg/kg) markedly decreased the mucosal PGE₂ content of the small intestine from 26.4 ± 4.4 ng/g tissue to less than 2 ng/g tissue within 3 h, and the values remained lowered during a 24-h test period (Fig. 2). Although SC-560 at 10 mg/kg decreased the mucosal PGE₂ content as effectively as indomethacin when determined 3 h after the administration, this effect was slightly but significantly recovered from 6 h after the treatment, and the level was almost totally restored to the basal value 12 h thereafter. However, the combined administration of SC-560 and rofecoxib significantly decreased the mucosal PGE₂ content at 12 h after the administration, compared with the control, the value being 6.0 ± 1.4 ng/g tissue, which is about 20% of the control value. Rofecoxib by itself had no effect on the mucosal PGE₂ content of the small intestine when determined at either 3 or 12 h after the administration.

As demonstrated in our previous study (Tanaka et al., 2002), we observed the expression of COX-2 mRNA in the intestinal mucosa 6 h after administration of indomethacin or SC-560 but not rofecoxib, although the gene expression of COX-2 was negligible in the normal rat intestine (Fig. 3). In contrast, both G3PDH and COX-1 mRNAs were observed in the intestinal mucosa of rats, irrespective of whether they were treated with vehicle, indomethacin, SC-560, or rofecoxib.

**Inflammatory Mucosal Responses to Various COX Inhibitors’ MPO Activity.** The MPO activity was 0.06 ± 0.01 µmol of H₂O₂/mg of protein in the normal intestinal mucosa and markedly elevated in response to indomethacin, reaching 0.16 ± 0.03 µmol of H₂O₂/mg of protein 24 h later (Fig. 4). Treatment of the animals with SC-560 or rofecoxib alone did not increase MPO activity in the intestinal mucosa, yet the combined administration of SC-560 plus rofecoxib significantly increased the MPO activity compared with control values observed in normal rats.

**iNOS Activity.** The iNOS activity in the normal intestinal mucosa was 0.06 ± 0.06 pmol/min/mg of protein (Fig. 5).

![Fig. 1. Intestinal ulcerogenic properties of various COX inhibitors in rats. The animals were administered indomethacin (10 mg/kg), SC-560 (10 mg/kg), rofecoxib (10 mg/kg), or SC560 plus rofecoxib p.o., and they were killed 24 h later. Data are presented as the mean ± S.E. from five to six rats.](image1)

![Fig. 2. Effects of various COX inhibitors on mucosal PGE₂ content of the rat small intestine. The animals were administered indomethacin (10 mg/kg), SC-560 (10 mg/kg), rofecoxib (10 mg/kg), or SC560 plus rofecoxib p.o., and killed 3, 6, or 12 h later. Mucosal PGE₂ content was measured by EIA. Data are presented as the mean ± S.E. from five to six rats. Significant difference from vehicle at *P < 0.05.](image2)
Indomethacin markedly increased iNOS activity in the intestinal mucosa when determined 24 h later, the value reaching about 8 times the basal level and being $0.36 \pm 0.11$ pmol/min/mg of protein. Neither SC-560 nor rofecoxib had any effect on Ca$^{2+}$-independent NOS activity, and the values in both cases were not significantly different from those observed in control rats. However, the iNOS activity was significantly increased in the animals treated with SC-560 plus rofecoxib, the value being $0.41 \pm 0.12$ pmol/min/mg of protein.

**iNOS Expression.** Reverse-transcription PCR analysis revealed that iNOS mRNA was expressed in the intestinal mucosa 6 h after administration of indomethacin, although it was not detected in the control mucosa (Fig. 6). Up-regulation of iNOS mRNA expression in the intestinal mucosa was similarly observed in the animals given SC-560 but not rofecoxib.

**Enterobacterial Invasion.** The number of aerobic and anaerobic enterobacteria in the normal intestinal mucosa was $6.73 \pm 0.18$ and $6.97 \pm 0.14$ log CFU/g tissue, respectively (Table 2). After subcutaneous administration of 10 mg/kg indomethacin, both values were markedly increased compared with controls, being $8.07 \pm 0.03$ and $8.25 \pm 0.18$ log CFU/g tissue, respectively. Likewise, SC-560 (10 mg/kg) also significantly increased the number of enterobacteria in the mucosa, the values for both aerobic and anaerobic bacteria being equivalent to those observed in indomethacin-treated animals. In contrast, the bacterial count in the mucosa remained unchanged after 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. The combined administration of SC-560 and rofecoxib also enhanced the number of enterobacteria, but the bacterial count was not further increased compared with that observed in the group given SC-560 alone.

**Effects of Various COX Inhibitors on Intestinal Motility.** Because intestinal hypermotility has been implicated as one of the pathogenic factors in NSAID-induced small intestinal lesions (Kunikata et al., 2002b; 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 after intraduodenal administration of indomethacin (10 mg/kg), in regard to both the amplitude and frequency of contraction (Figs. 7 and 8). Likewise, 10 mg/kg SC-560 also caused intestinal hypermotility, similar to indomethacin, and this effect persisted for over 3 h. In contrast, rofecoxib (10 mg/kg) did not have any
expression, up-regulates COX-2 expression, and the PGE2 ing intestinal hypermotility, bacterial invasion, and iNOS
2002) and furthermore that COX-1 inhibition, despite caus-
son et al., 1998; Konaka et al., 1999). The present study
duction (Fang et al., 1977; Robert and Asano, 1977; Bjarna-
SC-560/H11006

**TABLE 2**

Changes in enterobacterial number in the rat intestinal mucoasa after administration of various COX inhibitors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>No. of Rats</th>
<th>No. of Enterobacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td></td>
<td>Aerobic</td>
</tr>
<tr>
<td>Vehicle</td>
<td>6</td>
<td>6</td>
<td>6.73 ± 0.18</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>6</td>
<td>8.07 ± 0.03a</td>
</tr>
<tr>
<td>SC-560 (S)</td>
<td>10</td>
<td>6</td>
<td>8.41 ± 0.40a</td>
</tr>
<tr>
<td>Rofecoxib (R)</td>
<td>10</td>
<td>6</td>
<td>6.60 ± 0.32</td>
</tr>
<tr>
<td>S + R</td>
<td>10+10</td>
<td>6</td>
<td>8.12 ± 0.14a</td>
</tr>
</tbody>
</table>

* Significant difference from values for vehicle at *P* < 0.05.

*Fig. 7.* Representative recordings showing the effects of various COX inhibitors on intestinal motility in rats. Indomethacin (10 mg/kg), SC-560 (10 mg/kg), or rofecoxib (10 mg/kg) was given i.d. after basal intestinal motility had well stabilized. Quantitation of intestinal motility was done using NIH Image 1.61. Data were expressed as a motility index (arbitrary units) and are presented as the mean ± S.E. from four to six rats. Significant difference from vehicle at *, *P* < 0.05.

intestinal damage occurs only when both COX-1 and COX-2 are inhibited.

First, the present study confirmed that neither the selective COX-1 inhibitor SC-560 nor the selective COX-2 inhibitor rofecoxib damaged the small intestine, although mucosal PG levels were reduced by the former as effectively as indomethacin the nonselective COX inhibitor. However, the combined administration of the selective COX-1 and COX-2 inhibitors caused intestinal lesions, consistent with our previous observation (Tanaka et al., 2002). Because similar results in the stomach have been reported by other investigators (Wallace et al., 2000; Gretzer et al., 2001; Tanaka et al., 2001), it is considered that the adverse reaction of NSAIDs in the gastrointestinal tract is not accounted for solely by the inhibition of COX-1 and requires inhibition of COX-2 as well. In addition, we also confirmed that COX-2 mRNA was expressed after administration of SC-560 as well as indomethacin but not rofecoxib. Indeed, the mucosal 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. This represents a compensatory response to inhibition of PG biosynthesis and may explain why inhibition of both COX-1 and COX-2 is required for the ulcerogenic action of NSAIDs.

Second, we clearly showed in this study the role of COX-1 and COX-2 inhibition in various events responsible for NSAID-induced intestinal damage, including intestinal hypermotility, enterobacterial invasion, and an increase of MPO as well as iNOS activity (Takeuchi et al., 2002). All these events were reproduced in the animals after administration of indomethacin. Of interest, SC-560 also caused an increase in intestinal motility and the number of enterobacteria in the mucosa, suggesting a role for COX-1 inhibition in intestinal hypermotility in response to NSAID as well as a causal relationship between the hypermotility and enterobacterial invasion. Indeed, we previously reported that atropine, an anticholinergic drug, inhibits intestinal hypermotility induced by indomethacin, resulting in suppression of bacterial invasion as well as other inflammatory changes in

**Discussion**

It is recognized that conventional NSAIDs, which nonselectively inhibit both COX-1 and COX-2, damage the small intestine, concomitant with a decrease in mucosal PGE2 production (Fang et al., 1977; Robert and Asano, 1977; Bjarnason et al., 1998; Konaka et al., 1999). The present study confirmed that inhibition of both COX-1 and COX-2 is required for induction of intestinal damage (Tanaka et al., 2002) and furthermore that COX-1 inhibition, despite causing intestinal hypermotility, bacterial invasion, and iNOS expression, up-regulates COX-2 expression, and the PGE2 produced by COX-2 may counteract subsequent events such as increases in MPO and iNOS activity, and maintain the mucosal integrity. This sequence of events may explain why

![Fig. 8. Effects of various COX inhibitors on intestinal motility in rats. Indomethacin (10 mg/kg), SC-560 (10 mg/kg), or rofecoxib (10 mg/kg) was given i.d. after basal intestinal motility had well stabilized. Data were expressed as a motility index (arbitrary units) and are presented as the mean ± S.E. from four to six rats. Significant difference from vehicle at *, *P* < 0.05.](https://aspetjournals.org/doi/10.1124/jpet.2002.259.1252)
the small intestine (Kunikata et al., 2002b; Takeuchi et al., 2002). It has been reported that bacterial endotoxin enhances the intestinal permeability through expression of iNOS and overproduction of NO in the mucosa (Boughton-Smith et al., 1993; Whittle et al., 1995). This is supported by the present finding that indomethacin up-regulated iNOS expression with a concomitant increase in iNOS activity. The expression of iNOS mRNA was also observed in the intestinal mucosa after administration of SC-560 but not rofecoxib, indicating that the up-regulation of iNOS is associated with the inhibition of COX-1. This is understandable, because iNOS expression is triggered by endotoxin released from enterobacteria (Boughton-Smith et al., 1993) and because bacterial invasion is causally related with intestinal hypermotility due to COX-1 inhibition (Kunikata et al., 2002b; Takeuchi et al., 2002). However, SC-560 did not increase the iNOS activity in the mucosa, despite up-regulating iNOS mRNA expression, and a significant increase in this activity was observed when SC-560 was given together with rofecoxib. Because we previously reported the up-regulation of iNOS mRNA and NO production in the small intestine 6 h after administration of indomethacin (Tanaka et al., 1999), it is no doubt that the up-regulation of iNOS mRNA is followed by the iNOS protein expression, resulting in NO production in the small intestine. In addition, we also reported that the severity of indomethacin-induced intestinal damage was significantly reduced by aminoguanidine the relatively selective iNOS inhibitor as well as 16,16-dimethyl PGE2 given 6 h after administration of indomethacin (Tanaka et al., 1999, 2002). These results suggest that PGE2 may inhibit the iNOS activity similar to aminoguanidine. Alternatively, it is also possible that PGE2 regulates the post-transcriptional regulatory mechanisms to decrease iNOS protein expression or increase the protein degradation. On the other hand, Kobayashi et al. (2001) recently reported that COX-2 down-regulated iNOS expression in rat intestinal epithelial cells. In our study, SC-560 up-regulated COX-2 expression in the intestinal mucosa, probably due to inhibition of COX-1, and under such conditions the expression of iNOS mRNA was observed. The reason for the different results remains unknown, although the experimental conditions did differ between these two studies. Further study is needed to verify this point.

We recently reported that conventional NSAIDs at an ulcerogetic dose caused a marked hypermotility in the rat small intestine; in all cases, the motility change occurred within 20 to 30 min, much sooner than the onset of bacterial invasion and other inflammatory changes as well as development of intestinal damage. Because abnormal contraction of the intestinal wall results in disruption of the unstirred mucus layer over the epithelium, leading to increased mucosal susceptibility to pathogens and irritants, the intestinal hypermotility may play a role in the pathogenic mechanism of indomethacin-induced small intestinal lesions. Indeed, atropine similar to 16,16-dimethyl PGE2 potently inhibited intestinal hypermotility as well as bacterial invasion and other inflammatory changes in response to indomethacin, resulting in prevention of intestinal damage (Kunikata et al., 2002a,b). In the present study, we found that SC-560 but not rofecoxib induced intestinal hypermotility as well as enterobacterial invasion, similar to indomethacin. These findings suggest that the intestinal hypermotility induced by NSAIDs is related to a deficiency of PG caused by inhibition of COX-1, leading to enhancement of bacterial invasion in the mucosa.

Wallace et al. (2000) reported that SC-560, but not celecoxib, produced a decrease in gastric mucosal blood flow, suggesting that the effect of NSAIDs on the mucosal blood flow is brought about by suppression of COX-1. This may be compatible with the present finding that intestinal hypermotility was induced by inhibition of COX-1 but not COX-2 activity, because intestinal hypermotility caused mucosal hypoxia and microvascular injury due to smooth muscle contraction (Anthony et al., 1993, 1997). It has also been shown that the selective COX-2 inhibitor celecoxib increased neutrophil adherence in mesenteric venules similar to indomethacin, whereas the selective COX-1 inhibitor SC-560 did not (Wallace et al., 2000). In the present study, we found that intestinal MPO activity was increased when both COX-1 and COX-2 were inhibited by the combined administration of SC-560 plus rofecoxib. Neither SC-560 nor rofecoxib alone significantly increased the MPO activity in the intestinal mucosa. We previously reported that the increased MPO response to indomethacin was suppressed by ampicillin as well as atropine, suggesting that this event is closely associated with enterobacterial invasion (Kunikata et al., 2002a,b). In agreement with our previous findings (Tanaka et al., 2002), inhibition of COX-1 by SC-560 up-regulated COX-2 expression, which increased PGE2 production from 6 h after the administration, as a compensatory response to suppression of the biosynthesis of PG by COX-1 inhibition. Considering these results, it is assumed that inhibition of COX-2 may be related with the increase in MPO activity after the combined administration of SC-560 plus rofecoxib. It is known that neutrophils play a permissive role in NSAID-induced intestinal damage, inasmuch as these lesions were significantly prevented by antineutrophil serum (Konaka et al., 1999). 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 lesion model (Beckman et al., 1990). Thus, it may be assumed that COX-2 contributes to maintaining the integrity of the intestinal mucosa through inhibition of neutrophil migration under inhibition of COX-1.

In conclusion, the present results together suggest that inhibition of COX-1, despite causing intestinal hypermotility, bacterial invasion, and iNOS expression, up-regulates COX-2 expression, and the PGE2 produced by COX-2 may counteract subsequent events such as increases in MPO and iNOS activity and maintain the mucosal integrity. This sequence of events related to COX-1 or COX-2 inhibition may explain why intestinal damage occurs only when both COX-1 and COX-2 are inhibited. Finally, the present findings suggest a role for COX-2 as well as COX-1 in maintaining the integrity of the small intestinal mucosa and strongly indicate that inhibition of both COX-1 and COX-2 is required for NSAID-induced small intestinal damage.

References


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