Up-Regulation of Cyclooxygenase-2 by Inhibition of Cyclooxygenase-1: A Key to Nonsteroidal Anti-Inflammatory Drug-Induced Intestinal Damage

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) induce gastrointestinal ulceration as the adverse reaction. This effect of NSAIDs is attributable to endogenous prostaglandin (PG) deficiency caused by inhibition of cyclooxygenase (COX), yet the relation between COX inhibition and the gastrointestinal ulcerogenic property of NSAIDs remains controversial. Using selective COX inhibitors, we examined whether inhibition of COX-1 or COX-2 alone is sufficient for induction of intestinal damage in rats. Various COX inhibitors were administered p.o. in rats, and the animals were killed 24 h later. Mucosal PGE2 levels were determined by enzyme immunoassay, whereas the gene expression of COX isozymes was examined by reverse transcription-polymerase chain reaction. Nonselective COX inhibitors such as indomethacin inhibited PGE2 production and caused damage in the small intestine. Selective COX-2 inhibitors (rofecoxib or celecoxib) had no effect on the generation of PG, resulting in no damage. A selective COX-1 inhibitor (SC-560) did not cause damage, despite reducing PGE2 content. However, the combined administration of COX-1 and COX-2 inhibitors provoked intestinal damage with an incidence of 100%. COX-2 was up-regulated in the small intestine after administration of SC-560, and the PGE2 content was restored 6 h later, in a rofecoxib-dependent manner. The intestinal lesions induced by SC-560 plus rofecoxib were significantly prevented by later administration of 16,16-dimethyl PGE2. These results suggest that the intestinal ulcerogenic property of NSAID is not accounted for solely by inhibition of COX-1 and requires inhibition of COX-2 as well. The inhibition of COX-1 up-regulates COX-2 expression, and this may be a key to NSAID-induced intestinal damage.

After short-term and long-term administration, nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin cause intestinal ulceration in human and laboratory animals (Robert and Asano, 1977; Fang et al., 1977; Bjarnason et al., 1987). Although several factors have been postulated as the pathogenetic element of intestinal ulceration induced by indomethacin, including a deficiency of prostaglandins (PGs), bile acid, and bacterial flora (Whittle, 1981; Weissenborn et al., 1985; Asako et al., 1992; Yamada et al., 1993), the exact mechanisms remain unexplored. It is, however, believed that PG deficiency plays a critical role in the pathogenesis of these lesions.

The PG deficiency caused by NSAIDs is brought about by inhibition of cyclooxygenase (COX) activity. There are two isoforms of COX; COX-1 is constitutively expressed in various tissues, including the stomach, whereas COX-2 does not appear to be expressed in most tissues and is rapidly up-regulated in response to growth factors and cytokines (Feng et al., 1993; Kargman et al., 1993; O'Neill and Ford-Hutchinson, 1993). This tissue specificity has led to the contention that COX-1 is critical for housekeeping in the gastrointestinal mucosa (Chan et al., 1995; Ikari et al., 1999), whereas COX-2 is responsible for inflammation (Xie et al., 1992; Seibert et al., 1994; Langenbach et al., 1995). Indeed, studies using selective COX-2 inhibitors showed that the ulcerogenic property of NSAIDs is brought about by inhibition of COX-1 but not COX-2 (Putaki et al., 1993; Chan et al., 1995). However, this paradigm has been challenged by recent studies (Wallace et al., 2000; Gretzer et al., 2001; Takeuchi et al., 2001), and the relation between the inhibition of COX-1 and gastric ulcerogenic effect of NSAIDs remains controversial. Wallace et al. (2000) showed that inhibition of both COX-1 and COX-2 is required for NSAID-induced gastric injury, suggesting a role for COX-2 as well as COX-1 in maintaining the integrity of the stomach. Gretzer et al. (2001) also reported a protective role for COX-2 in the stomach after acid challenge. Furthermore, Langenbach et al. (1995) reported that COX-1 knockout mice do not spontaneously develop...
gastric lesions, as further evidence that inhibition of COX-1 alone is not sufficient to induce gastric damage. However, no study has been reported on the relation of COX inhibition and the intestinal ulcerogenic property of NSAIDs.

In the present study, we evaluated the ulcerogenic effect of nonselective COX inhibitors as well as selective inhibitors of COX-1 (SC-560) (Smith et al., 1998) and COX-2 (rofecoxib and celecoxib) (Chan et al., 1995; Warner et al., 1999; Wallace et al., 2000) in the rat small intestine and investigated whether the inhibition of COX-1 is sufficient by itself to cause intestinal damage. In addition, we also investigated why inhibition of both COX-1 and COX-2 is required for the ulcerogenic action of NSAIDs in the rat small intestine.

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 here were approved by the Experimental Animal Research Committee of the Kyoto Pharmaceutical University.

Evaluation of Small Intestinal Ulcerogenic Property. The animals were treated orally by esophageal intubation with the NSAID (nonselective COX inhibitor) indomethacin (10 mg/kg), flurbiprofen (20 mg/kg), naproxen (40 mg/kg), or diclofenac (40 mg/kg), the selective COX-1 inhibitor SC-560 (10–100 mg/kg), and the selective COX-2 inhibitor rofecoxib (10–100 mg/kg) or celecoxib (30–300 mg/kg) and were killed 24 h after the administration, under deep ether anesthesia. In a separate study, the ulcerogenic effect on the small intestine of the combined administration of SC-560 and rofecoxib was examined. The animals were treated orally with SC-560 (1–10 mg/kg) in combination with rofecoxib (10 mg/kg), with rofecoxib (1–10 mg/kg) in combination with SC-560 (10 mg/kg), with celecoxib (30 mg/kg) in combination with SC-560 (10 mg/kg), and were killed 24 h after these treatments. In some cases, 16,16-dimethyl prostaglandin E₂ (dmPGE₂) 1–10 μg/kg) was given p.o. 6 h after the combined administration of SC-560 and rofecoxib. In each case, to delineate the damage 1 ml of Evans blue (w/v) was injected i.v. 30 min before sacrifice (Konaka et al., 1999a). The small intestines were excised and treated with 2% formalin for fixation of the tissue walls. Then, they were opened along the anti-mesenteric attachment and examined for damage under a dissecting microscope with square grids (√x10). The area (mm²) 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 PGE₂ Contents. PGE₂ levels in the small intestinal mucosa were measured after p.o. administration of various NSAIDs (10 mg/kg indomethacin, 20 mg/kg flurbiprofen, 40 mg/kg naproxen, and 40 mg/kg diclofenac) and selective COX-1 or COX-2 inhibitors (10 mg/kg SC-560, 10 mg/kg rofecoxib, and 30 mg/kg celecoxib). In most cases, the animals were killed under deep ether anesthesia 3 h after the administration, and the small intestinal tissue was isolated, weighed, and put in a tube containing 99.8% methanol plus 0.1 M indomethacin (Putaki et al., 1994). The tissues were then homogenized by 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 resolved in assay buffer and used for determination of PGE₂. The concentration of PGE₂ was measured using a PGE₂ enzyme immunoassay (EIA) kit (Amersham Pharmacia Biotech, U.K.). In some cases, the mucosal PGE₂ content was measured at 3, 6, 12, and 24 h after administration of 10 mg/kg indomethacin or 10 mg/kg SC-560, and at 12 h after the combined administration of 10 mg/kg SC-560 plus 10 mg/kg rofecoxib.

Analysis of COX-1 and COX-2 mRNA by Reverse Transcription-Polymerase Chain Reaction. The animals were killed under deep ether anesthesia at various time points (0, 3, and 6 h) after administration of SC-560 (10 mg/kg) and at 6 h after administration of indomethacin (10 mg/kg) or rofecoxib (10 mg/kg), and the 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 hexadeoxy ribonucleotide was reverse-transcribed with SUPER-SCRIPT preamplification system (Invitrogen). The sequences of sense and antisense primers for the rat COX-1 were 5’-AACCG TGTGCTGACCTTGCTGAA-3’ and 5’-AAAGAGGCCTCAGGAGCTCAG TG-3’, respectively, giving rise to a 887-bp PCR product (Feng et al., 1993). For the rat COX-2, the sequences of sense and antisense primers were 5’-TGTGACTGCCA ACTCCCATG-3’ and 5’-AATGTTGAAGGTGTCGGACG-3’, respectively, giving rise to a 702-bp PCR product (Iso et al., 1995). For the rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a constitutively expressed gene, the sequences were 5’-GAAACGGGAAGCTCAGGGCAT- GCC-3’ for the sense primer and 5’-TGAGG TCCACACCTGGTGTGCTG-3’ for the antisense primer, giving rise to a 310-bp PCR product (Feng et al., 1993). An aliquot of the RT 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.

Immunostaining of COX-1 and COX-2. Immunostaining of COX isozymes in the intestinal mucosa was performed 6 h after administration of SC-560 (10 mg/kg, p.o.). The intestines were removed and washed in phosphate-buffered saline. The specimens were put in embedding medium (O.C.T. compound; Sakura Fine-technical Co., Ltd., Tokyo, Japan) and rapidly frozen. Cryostat sections cut serially at a thickness of 12 μm were mounted on silanized slides and stained with rabbit polyclonal antibodies against rat COX-1 and COX-2 (both from Santa Cruz Biotechnol Inc., Santa Cruz, CA), each diluted 1: 200 in phosphate-buffered saline. Immunohistochemical staining was performed by a streptavidin-biotin peroxidase method, according to the manufacturer’s instructions (rabbit ABC Staining System; Santa Cruz Biotechnology Inc.).

Preparation of Drugs. Drugs used were indomethacin, naproxen, diclofenac, flurbiprofen (Sigma, St. Louis, MO), SC-560 (Cayman Chemical, Ann Arbor, MI), celecoxib and rofecoxib (synthesized in our laboratory), 16,16-dimethyl PGE₂ (dmPGE₂; Funakoshi, Tokyo, Japan), and Evans blue (Merck, Darmstadt, Germany). All NSAIDs and COX-inhibitors were suspended in hydroxypropylcellulose solution. dmPGE₂ was first dissolved in absolute ethanol and then diluted to desired concentrations with saline. Other drugs were dissolved in saline. All drugs were prepared immediately before use and administered p.o. in a volume of 0.5 ml/100 g body weight or i.v. in a volume of 0.1 ml/100 g body weight.

Statistics. Data are presented as the mean ± S.E. of six to four 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 NSAIDs and COX Inhibitors on the Intestinal Mucosa and PGE₂ Content. Oral administration of nonselective COX inhibitors provoked hemorrhagic damage in the small intestinal mucosa within 24 h, mainly in the jejunum and ileum; the lesion score being 215.6 ± 15.2
mm², 148.3 ± 14.7 mm², 217.1 ± 22.4 mm², and 181.3 ± 24.6 mm², respectively, for 10 mg/kg indomethacin, 40 mg/kg dicrofenac, 20 mg/kg flurbiprofen, and 40 mg/kg naproxen (Fig. 1A). The apparent size and morphology of intestinal lesions were very much similar, irrespective of which NSAID was used to induce the damage. However, neither the selective COX-1 inhibitor SC-560 (10–100 mg/kg) nor the selective COX-2 inhibitors rofecoxib (10–100 mg/kg) and celecoxib (30–300 mg/kg) induced any gross damage in the small intestine during the same test period (Fig. 2A).

On the other hand, all nonselective COX inhibitors at the doses used caused a marked decrease in the mucosal PGE² content of the small intestine (Fig. 1B). The mucosal PGE² content (25.0 ± 4.6 ng/g tissue) had decreased by 90% 3 h after the administration of these agents. Likewise, SC-560 (10 mg/kg) also caused a significant decrease in PGE² content, and the inhibitory effect on PGE² production was equivalent to that induced by indomethacin (10 mg/kg). However, the selective COX-2 inhibitors rofecoxib (10 mg/kg) and celecoxib (30 mg/kg) had no effect on the mucosal PGE² content of the small intestine (Fig. 2B).

**Effect of Combined Treatment with SC-560 and Rofecoxib on the Small Intestinal Mucosa.** To further investigate the role of COX-1 and/or COX-2 inhibition in the development of small intestinal damage, we examined the ulcerogenic response to the combined p.o. administration of SC-560 and rofecoxib or celecoxib in the small intestine. Again, the selective COX-2 inhibitors rofecoxib (10 mg/kg) and celecoxib (30 mg/kg) did not damage the small intestine (Fig. 3A). Likewise, the selective COX-1 inhibitor SC-560 at 10 mg/kg also did not cause intestinal damage. However, SC-560 did produce hemorrhagic lesions in the small intestine at an incidence of 100% when administered together with rofecoxib or celecoxib, the lesion score being 89.1 ± 7.2 and 103.1 ± 28.4 mm², respectively. These lesions induced by the combined administration of SC-560 plus rofecoxib looked very much similar to those induced by conventional NSAIDs, concerning the site of occurrence as well as the apparent size and morphology. Furthermore, when SC-560 at the nonulcerogenic dose (10 mg/kg) was given together with the COX-2 inhibitor rofecoxib (1–10 mg/kg), damage was observed dependent on the dose of rofecoxib (Fig. 3B). The same was
observed for SC-560 (1–10 mg/kg) when administered in the presence of rofecoxib (10 mg/kg). However, when SC-560 (10 mg/kg) or rofecoxib (10 mg/kg) alone was administered repeatedly twice every 6 h and then the animals were killed 24 h after the first injection, there was no damage to the small intestine (not shown).

Expression of COX-1 and COX-2 mRNAs in the Intestinal Mucosa after Administration of Various COX Inhibitors. Although the gene expression of COX-2 was negligible in the normal rat intestine, the expression of COX-2 mRNA was found to be up-regulated in the rat small intestine in provoking mucosal damage in the rat small intestine. The animals were administered SC-560 (1–10 mg/kg) and rofecoxib (1–10 mg/kg) p.o., in combination at different doses, and killed 24 h later. Data are presented as the means ± S.E. from five to six rats.

Time Course of Changes in the PGE₂ Content of the Intestinal Mucosa after Administration of Various COX Inhibitors. Oral administration of indomethacin (10 mg/kg) markedly decreased the mucosal PGE₂ content of the small intestine from 31.1 ± 6.0 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. 6). 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 almost totally restored to the basal values 12 h thereafter. The PGE₂ content 12 h and 24 h after treatment with SC-560 was 22.5 ± 5.3 ng/g tissue and 24.9 ± 4.8 ng/g tissue, respectively, neither of which was significantly different from the basal value.

To further investigate whether the recovery of PGE₂ production at 12 h after administration of SC-560 is due to COX-1 or COX-2, we measured the mucosal PGE₂ content 12 h after the combined administration of SC-560 and rofecoxib. Neither SC-560 (10 mg/kg) nor rofecoxib (10 mg/kg) by
itself had any effect on the mucosal PGE2 content of the small intestine when determined at 12 h after the administration (Fig. 7). However, the combined administration of SC-560 and rofecoxib significantly decreased the mucosal PGE2 content as compared with the control, the value being 6.0 ± 1.4 ng/g tissue, which is about 20% of the control value. The recovery of PGE2 contents at 12 h after administration of
SC-560 was similarly attenuated by subsequent administration of rofecoxib but not SC-560, given 6 h after the first dosing of SC-560 (not shown).

**Effect of dmPGE₂ on Intestinal Damage Induced by the Combined Administration of SC-560 and Rofecoxib.** To investigate whether the COX-2-derived PGE₂ production plays a critical role in the onset of intestinal damage after the combined administration of COX-1 and COX-2 inhibitors, we examined the effect of a later dosing of dmPGE₂ after the combined administration of SC-560 and rofecoxib. Consistent with previous studies (Whittle, 1981; Konaka et al., 1999a; Takeuchi et al., 2001), the present study showed that the selective COX-1 inhibitor, SC-560, yet gradually recovered from 6 h after the administration of these agents, the selective COX-1 and COX-2 inhibitors, provoked damage in the small intestine. Wallace et al. (2000) recently reported that neither SC-560 nor celecoxib induced gastric lesions in rats, suggesting that inhibition of both COX-1 and COX-2 is required for the occurrence of NSAID-induced gastric injury. The present findings together with those data indicate that COX-2 plays a “housekeeping” role in the gastrointestinal mucosa and that the adverse reaction of NSAIDs is not accounted for solely by inhibition of COX-1.

The most important finding in this study is that inhibition of COX-1 by SC-560 up-regulated the expression of COX-2 mRNA in the intestinal mucosa. We have previously reported that the gene expression of COX-2 was induced in the gastric mucosa after administration of both indomethacin and SC-560 (Tanaka et al., 2001b). Our results also confirmed that COX-1 is expressed in the normal intestinal mucosa including the epithelial cells and the lamina propria, yet COX-2 is scanty. In the intestinal mucosa of indomethacin-treated rats, however, there were a number of epithelial cells that stained positively with the COX-2 antibody, supporting the theory that COX-2 is expressed in the intestine of such animals. The COX-2 expression in epithelial cells has been reported by Singer et al. (1998), who showed that COX-2 is induced in apical epithelial cells of inflamed foci in inflammatory bowel diseases. As expected, 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. A rapid up-regulation of COX-2 expression after inhibition of COX-1 may represent a compensatory response to inhibition of PG biosynthesis and contributes to maintenance of the mucosal integrity under such conditions. This speculation was supported by the findings that 1) additional treatment with rofecoxib and SC-560 attenuated the later production of PGE₂ due to COX-2 and provoked damage in the small intestine, and 2) later administration of dmPGE₂ significantly prevented the occurrence of intestinal damage in response to the combined treatment with SC-560 plus rofecoxib. Although the gene expression of COX-2 was also observed in the mucosa after administration of indomethacin, there was no recovery of PGE₂ because of inhibition of COX-2 activity.

**Discussion**

It is recognized that conventional NSAIDs damage the gastrointestinal mucosa in experimental animals and humans. These NSAIDs inhibit nonselectively the COX activity, irrespective of the type of COX isozyme, i.e., COX-1 or COX-2, but the relation of these COX isozymes to the ulcogenic property remains unclear. The present study confirmed that conventional NSAIDs, which nonselectively inhibit both COX-1 and COX-2, produced damage in the small intestine, concomitant with a decrease in mucosal PGE₂ production (Whittle, 1981; Konaka et al., 1999a; Tanaka et al., 1999; Takeuchi et al., 2001). In addition, we also found that although neither the selective COX-1 nor COX-2 inhibitor alone caused gross damage in the intestinal mucosa, their combination did provoke apparent damage in the small intestine, similar to conventional NSAIDs. Furthermore, we showed for the first time, to our knowledge, that inhibition of COX-1 up-regulated the COX-2 expression in the intestinal mucosa, which may explain the lack of an intestinal ulcogenic effect by the selective COX-1 inhibitor.
by this agent, leading to intestinal damage. At present, the exact mechanism by which COX-2 is up-regulated after inhibition of COX-1 remains unknown. Because enterobacteria play a key pathogenic role in the formation of NSAID-induced intestinal lesions, through release of lipopolysaccharide and expression of inducible nitric oxide synthase (iNOS) (Boughton-Smith et al., 1993; Whittle et al., 1995; Konaka et al., 1999b), it is possible that COX-2 is up-regulated by lipopolysaccharide, similar to iNOS in the mucosa. Alternatively, because NSAIDs reportedly release tumor necrosis factor-α as an early event in the formation of intestinal lesions (Bertrand et al., 1998), the up-regulation of COX-2 observed under COX-1 inhibition is mediated by tumor necrosis factor-α. Further studies are needed to verify these points.

How inhibition of both COX-1 and COX-2 leads to development of intestinal lesions remains unknown. Wallace et al. (2000) recently 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. The same authors also showed that the selective COX-2 inhibitor celecoxib increased with that achieved with indomethacin, whereas the selective COX-1 inhibitor SC-560 did not. Neutrophils also play a permissive role in NSAID-induced intestinal lesions, inasmuch as these lesions were significantly prevented by anti-neutrophil serum (Konaka et al., 1999b). These blood cells are the source of oxygen radicals and iNOS, and peroxynitrates formed by the interaction of NO with oxygen radicals may be detrimental in this lesion model (Beckman et al., 1990; Konaka et al., 1999a). It may be assumed that inhibition of COX-2 contributes to the process of intestinal damage through an increase of neutrophil activity. On the other hand, we have observed in a preliminary study that SC-560 by itself caused an increase of enterobacterial translocation and intestinal vascular permeability, whereas neutrophil activation was observed only after the combined administration of SC-560 plus rofecoxib (Tanaka et al., 2001a). Rofecoxib alone had no effect on any of these parameters. At present, just how enterobacterial translocation is enhanced under inhibition of COX-1 remains unknown.

Considering the results of the present study together with findings by others, one may speculate that conventional NSAIDs somehow produce bacterial translocation, leading to neutrophil activation and iNOS expression, and by so doing cause intestinal damage (Boughton-Smith et al., 1993; Yamada et al., 1993; Whittle et al., 1995; Konaka et al., 1999b). The bacterial translocation is associated with a deficiency of PG caused by inhibition of COX-1 (Tanaka et al., 2001a). However, inhibition of COX-1 up-regulates the expression of COX-2, and PGs produced by COX-2 may suppress the detrimental processes associated with COX-1 inhibition, including overproduction of iNOS/NO (Kobayashi et al., 2001; Tanaka et al., 2001a) or recruitment of neutrophils (Wallace et al., 2000). These sequential events related to COX-1 and/or COX-2 inhibition may explain why intestinal damage occurs only when both COX-1 and COX-2 are inhibited. Further study is certainly needed to verify this hypothesis.

In conclusion, the present study suggests that the intestinal ulcerogenic property of NSAIDs is not accounted for solely by COX-1 inhibition and requires the inhibition of both COX-1 and COX-2. The inhibition of COX-1 up-regulates the COX-2 expression, and this may counteract the deleterious influences of the PG deficiency caused by COX-1 inhibition. Finally, the present findings suggest a role for COX-2 as well as COX-1 in maintaining of the integrity of the small intestine, and strongly indicate that inhibition of both COX-1 and COX-2 is required for NSAID-induced small intestinal damage.

References
Beckman JS, Beekman TW, Chen-J, Marshall PA, and Freeman BA (1990) Apparent hydroxy radical production by peroxynitrite: implication for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87:1620–1624.


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