Title page.

Title:

Cyclooxygenase (COX)-1 and COX-2 both play an important role in the protection of the duodenal mucosa in cats

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2. Running title page.

a) Running title:

Role of COX in the protection of duodenal mucosa

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COX, cyclooxygenase; GI, gastrointestinal; IND, indomethacin; IR, immunoreactivity; TLA, total lesion area

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ABSTRACT

Though NSAIDs often cause ulcers in the duodenum in humans, the role of cyclooxygenase (COX) isoforms in the pathogenesis of duodenal ulcers has not been fully elucidated. We examined in cats the (1) ulcerogenic effects of selective COX-1 (SC-560, ketorolac) and COX-2 (celecoxib, meloxicam) inhibitors on the gastrointestinal mucosa, (2) effect of feeding and cimetidine on the expression of COX isoforms and PGE$_2$ level in the duodenum, and (3) localization of COX isoforms in the duodenum. COX inhibitors were administered after the morning meal in cats once daily for 3 days. Gastrointestinal lesions were examined on day 4. Localization and expression of COX isoforms (by immunohistochemistry, western blot) and PGE$_2$ level (by EIA) were examined. Results were as follows. (1) Selective COX-1 or COX-2 inhibitors alone produced marked ulcers in the duodenum but did not cause obvious lesions in the small intestine. Co-administration of SC-560 and celecoxib produced marked lesions in the small intestine. (2) Feeding increased both the expression of COX isoforms and PGE$_2$ level in the duodenum, and the effects were markedly inhibited by pretreatment with cimetidine. (3) COX-1 was localized in goblet and Brunner’s gland cells, Meissner’s and Auerbach’s plexus, smooth muscle cells, and arterioles; and COX-2 was observed in capillaries, venules and basal granulated cells. The expression of COX isoforms in the duodenum is up-regulated by feeding, and inhibition of either COX-1 or COX-2 causes ulcers in the duodenum, suggesting that both
isoforms play an important role in the protection of the duodenal mucosa.
Introduction

There are two isoforms of cyclooxygenase (COX) in the gastrointestinal (GI) tract; i.e., COX-1 which maintains the integrity of the GI mucosa under physiological conditions, and COX-2 which is implicated in invasive events such as inflammation (Michell et al., 1993; Masferrer et al., 1994; Takeuchi et al., 2010a). Therefore, it has been commonly believed that adverse effects on the GI tract of conventional nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin (IND) are caused by their inhibitory effect on COX-1 (Vane and Botting, 1995; Warner et al., 1999). This contention is based on the finding that selective COX-2 inhibitors such as celecoxib and rofecoxib neither decreased prostaglandin (PG) levels nor produced lesions in the GI mucosa in experimental animals (Futaki et al., 1993; Seibert et al., 1994; Laudanno et al., 2001). Morham et al. (1995) also reported that COX-2 deficiency did not cause spontaneous ulceration in the stomach in COX-2 knockout (KO) mice. Conversely, Langenbach et al. (1995) proposed a query on the role of COX-1 in maintaining the integrity of the gastric mucosa, since COX-1 KO mice were shown to have no damage to the stomach despite a marked decrease in gastric PG levels (< 1% of wild-type mice). Wallace et al. (2000) also reported that the selective COX-1 inhibitor SC-560 did not cause any visible lesions in the rat stomach despite a marked decrease in PG levels, but that the co-administration of SC-560 and celecoxib induced obvious lesions in the
stomach. Based on these results, it was proposed that simultaneous inhibition of both COX-1 and COX-2 is necessary for the formation of gastric lesions induced by NSAIDs. This notion was supported by studies (Gretzer et al., 2001; Tanaka et al., 2001) using SC-560 and the selective COX-2 inhibitor rofecoxib in a rat gastric lesion model. Similarly, inhibition of both COX isoforms is required to produce lesions in the small intestine in rats (Tanaka et al., 2002a). Sighthorsson et al. (2002) reported that short-term deficiency or inhibition of COX-1 or COX-2 alone did not cause any damage to the small intestine of mice, but that dual inhibition of COX isoforms led to damage similar to that seen with IND.

While the ability of NSAIDs to cause ulcers in the duodenum of humans is well known (Hawkey 1990; Allison et al., 1992), it has not been widely reported whether inhibition of both COX isoforms is necessary for induction of NSAID-induced duodenal ulcers, probably due to the lack of suitable animal models for duodenal ulcers induced by NSAIDs. Recently, we reported that the non-selective COX inhibitor IND produced obvious lesions in the duodenum of cats (Satoh et al., 2009). Therefore, in the present study, we examined in cats the 1) ulcerogenic effects of selective COX-1 (SC-560 and ketorolac) and COX-2 (celecoxib and meloxicam) inhibitors (Smith et al., 1998; Warner et al., 1999) on the GI mucosa, 2) effects of feeding and cimetidine on the expression of COX isoforms and the PGE$_2$ level in the duodenum, and 3) localization of COX isoforms in the duodenum. Our results showed that the expression of COX
isoforms in the duodenum is up-regulated by feeding, and that inhibition of either COX-1 or COX-2 alone causes lesions in the duodenal mucosa in cats, suggesting that both isoforms play an important role in the protection of the duodenal mucosa.
Materials and Methods

Ethics approval. Experimental protocols were approved by the Animal Research Committees, Division of Pathological Sciences, Kyoto Pharmaceutical University, Kyoto Japan.

Animals. Male and female mongrel cats bred for the experiments in the animal house of Kyoto Pharmaceutical University were used after feeding for >8 months. During the experiments, the animals were given dry food (Canet®, Petline, Gifu, Japan) twice daily between 9 - 10 AM and 5 - 6 PM.

Drugs. The following drugs were used: atropine sulfate (Tanabe, Tokyo, Japan), celecoxib (Celex®, Pfizer, Tokyo, Japan), cimetidine (Wako, Osaka, Japan), indomethacin (Wako), ketorolac (Sigma, St. Louis, MO, USA), meloxicam (Metacam®, Boehringer Ingelheim, Hyogo, Japan), pentobarbital sodium (Nembutal®, Dainippon-Sumitomo, Osaka, Japan), SC-560 (Alexis, Lausen, Switzerland), and xylazine (Ceractal®, Bayer, Tokyo, Japan). Drugs for subcutaneous injection were dissolved in physiological saline, while drugs for oral administration were suspended in 1% carboxymethylcellulose (CMC, Wako); 0.5 ml/kg of each drug was administered.

Antibodies. The primary antibodies used for immunohistochemistry and western blot analysis were a polyclonal rabbit anti-ovine COX-1 antibody (Cayman Chemical, Ann Arbor, MI,
USA) and a polyclonal anti-PGHS-2 (COX-2) human C-terminus (Oxford Biomedical Research, Oxford, MI, USA). These antibodies have been used in immunohistochemical studies in cats (Hayes et al., 2006).

**Macroscopic examinations of mucosal damage in the GI tract.** Each NSAID was administered p.o., except for meloxicam (s.c.), 1 h before or just after the morning meal once daily for 3 days, and the GI lesions were evaluated 24 h after the final dose of NSAIDs. Cats were sacrificed by bleeding from the carotid arteries under deep anesthesia with a combination of xylazine (2 mg/kg, i.m.), atropine (0.1 mg/kg, i.m.) and pentobarbital (25-50 mg/kg, i.v.) 24 h after the final dosing of NSAIDs. Then the whole GI tract was removed and cut along the longitudinal axis. The GI tract was spread out on paper and mucosal lesions in the stomach, duodenum and small intestine were observed macroscopically. The whole length of the small intestine from the duodenum to the ileum was divided into 10 parts of equal length, and the first part was regarded as the duodenum. The lesion area (cm²) was obtained from the product of the length and width of the lesions, and the total lesion area (TLA) was obtained by summing the area of individual lesions in each part of the GI tract. Though the duodenum is part of the small intestine, the TLA in the small intestine was calculated as the sum of the lesion areas in parts 2 to 10 of the small intestine. The proximal duodenum was removed, and immersed in 20% formalin for histological examination.
Histological examinations.

Staining with hematoxylin-eosin or PAS. Serial sections (4 μm) of formalin-fixed, paraffin-embedded samples of each tissue were mounted on slides. According to conventional methods, either hematoxylin and eosin or periodic acid-Schiff (PAS) staining was performed.

Measurement of the number of PAS-positive goblet cells in the villi. Photographs of the proximal duodenum stained with PAS in the normal control cats or in the cats given COX inhibitors were taken under a microscope (x40). Ten villi (>400 μm in length) were selected in each sample, and both the length of the villus and the number of PAS-positive cells were measured in each villus. The number of positive cells per 100-μm length of villus was calculated for each villus. The mean number of PAS-positive cells per 10 villi was then obtained for each cat.

Immunohistochemical staining. According to the method described by Hayes et al. (2006), localization of COX isoforms in the duodenum was examined in normal control cats. The animals were sacrificed as described above before (fasted) or just after a 1-h feeding period (fed). The proximal duodenum was removed, and immersed in 20% formalin. Serial sections (4 μm) of formalin-fixed, paraffin-embedded samples of each tissue were mounted on MAS coated glass slides. Antigen retrieval was performed at 90°C for 20 min in HistoVT One® Retrieval Solution (Nacalai Tesque, Kyoto, Japan). After deactivation of endogenous peroxidase with 0.3% H₂O₂
and blockade of nonspecific binding sites with 0.5% goat serum, the sections were reacted with anti-COX-1 or anti-COX-2 antibody 1:400 dilution overnight at 4°C. COX-1 and COX-2 proteins were visualized using a Vectastain ABC-peroxidase kit (Vector, Burlingame, CA, USA). Identical samples incubated with the primary antibody omitted were used as negative controls. Slides were counterstained with hematoxylin.

**Biochemical examinations.** In this study, 4 cats were used in each group, and duodenal samples were obtained before and 0.5 h after a 1-h feeding of diet after a 16-h fast. In another study, cimetidine (40 mg/kg, p.o.) was administered 1 h before feeding, and duodenal samples were obtained 0.5 h after a 1-h feeding period. The animals were sacrificed by bleeding from the carotid arteries under deep anesthesia as described above, and a 6-cm length of the proximal duodenum was removed from each animal and cut into 2 samples. The samples were either frozen in acetone/dry-ice and stored at -80 °C until western blotting, or transferred to a tube containing 99.8% methanol plus 0.1 M IND and stored at -80 °C until the assay of PGE$_2$ by EIA.

**Western blot analysis for COX-1 and COX-2.** The expression of COX proteins in the samples was assessed by western blot analysis as previously described (Takeuchi et al., 2010b). Samples were homogenized with protease inhibitor cocktail tablets (Complete, Roche, Penzberg, Germany) and centrifuged at 20,000 g for 30 min at 4°C. Supernatants were used for protein determination. The protein concentration of each sample was measured using a BCA protein
assay kit (Pierce, Rockford, IL, USA). Fifty micrograms of protein were electrophoresed on sodium lauryl sulfate (SDS)-12% polyacrylamide slab gels, and electrophoretically transferred to a PVDF membrane (Immu-No-Blot; Bio-Rad, Hercules, CA, USA). The membrane was incubated with primary antibody for COX-1, COX-2 or β-actin at 4°C overnight. The membranes were then incubated with anti-rabbit IgG horseradish peroxidase (HRP)-conjugated (Santa Cruz, Santa Cruz, CA, USA), or anti-mouse IgG-HRP (Santa Cruz) as a secondary antibody for 2 h. The immunoreactive bands were visualized by an enhanced chemiluminescence system (Western Blot Chemiluminescence Reagent Plus; NEN, Boston, MA, USA), and developed films were scanned and analyzed densitometrically. The integrated density of the bands was quantified using Quantity One analysis software. Values are expressed as the relative intensity of the mean density (per β-actin) for 4 fasted cats.

**Determination of PGE₂ levels.** PGE₂ levels in the duodenum were determined as previously described (Tanaka et al., 2002b). In brief, the duodenal samples were homogenized using a Polytron homogenizer and centrifuged at 10,000 g for 10 min at 4°C. After the supernatant of each sample had been evaporated under N₂ gas, the residue was resolved in assay buffer and used for determination of PGE₂. The concentration of PGE₂ in the sample of duodenal homogenates was measured using a PGE₂ EIA kit (Cayman Chemical). The protein concentrations of each sample were measured using BCA protein assay kits (Pierce, Rockford,
Statistics. All data are expressed as mean ± SEM. Differences between groups were analyzed by Student’s t-test for paired group comparisons, or analysis of variance (Dunnett’s multiple range test) if more than 2 variables were considered, with the significance level set at p<0.05.
Results

Effects of selective COX inhibitors on GI mucosa.

**Macroscopic observation.** In normal control cats (4 fasted and 4 fed), no lesions were observed in the GI tract. When COX inhibitors were administered after feeding of the diet, none of them produced visible lesions in the stomach.

**Effects of drugs given after feeding of diet.** SC-560, a selective COX-1 inhibitor, at doses of 3 and 10 mg/kg p.o. produced severe lesions in the duodenum in a dose-dependent manner, the TLAs in the duodenum were 0.4 ± 0.1 cm² (n=4) and 1.2 ± 0.1 cm² (n=4), respectively (Fig. 1). Two or 3 large lesions were often observed in the anterior and posterior regions of the proximal duodenum just caudal to the pyloric ring (Fig. 2A). SC-560 at a dose of 3 mg/kg did not cause any visible lesions in the small intestine, but at 10 mg/kg SC-560 caused mild lesions in the small intestine in 2 of the cats (Fig. 1). Another COX-1 inhibitor, ketorolac, at dose of 3 mg/kg p.o. produced marked lesions in the duodenum (TLA: 0.6± 0.3 cm², n=4) in all cats but did not cause lesions in the small intestine (Fig. 1).

Celecoxib, a selective COX-2 inhibitor, at dose of 10 mg/kg p.o. produced severe lesions in the duodenum in all 4 cats (Fig. 2B), and caused mild lesions in the small intestine in 1 cat. The TLAs in the duodenum and small intestine were 0.6 ± 0.2 cm² and 0.5 ± 0.3 cm² (n=4),
respectively. Another COX-2 inhibitor, meloxicam (0.6 mg/kg, s.c.), produced marked lesions in the duodenum in all 4 animals but did not cause any visible lesions in the small intestine (Fig. 1).

Effects of simultaneous inhibition of both COX-1 and COX-2. Co-administration of SC-560 (3 mg/kg, p.o.) and celecoxib (10 mg/kg, p.o.) caused severe lesions not only in the duodenum but also in the small intestine (Fig. 1). The TLAs in the duodenum and small intestine were $0.7 \pm 0.2$ cm$^2$ and $4.1 \pm 1.0$ cm$^2$ (n=4), respectively. The TLA of the small intestine was significantly (P<0.01) larger than that with administration of SC-560 or celecoxib alone (Fig. 1). As reported in our previous paper (Satoh et al., 2009), the non-selective COX inhibitor IND (3 mg/kg, p.o.) produced overt lesions in both the duodenum and the lower half of the small intestine in all 5 cats (Fig. 1). The TLAs in the duodenum and small intestine were $0.8 \pm 0.2$ cm$^2$ and $7.7 \pm 2.0$ cm$^2$ (n=5), respectively.

Effects of drugs given before feeding of diet. When a low dose (3 mg/kg) of SC-560 was administered 1h before the morning meal once daily for 3 days, it produced severe lesions in the duodenum. The TLAs in the duodenum and small intestine were $1.4 \pm 0.5$ cm$^2$ and $0.1 \pm 0$ cm$^2$ (n=4), respectively. The TLA of the duodenum was significantly (P<0.05) larger than that (0.5 ± 0.1 cm$^2$, n=4) caused by the same dose of SC-560 given after the morning meal (Table 1). A similar result was also observed in the duodenum by the administration of celecoxib, i.e., the TLA in the group given celecoxib (10 mg/kg, p.o.) before feeding was significantly (p<0.05) larger
than that given celecoxib after feeding (Table 1).

**Histological examination.** No pathological lesion was detected in the duodenal mucosa in normal control cats (Fig. 2C). The duodenal lesions caused by SC-560 or celecoxib had very similar changes, i.e., changes were observed just below the pyloric ring, and were UL-II grade ulcers, in which destruction of the *lamina muscularis mucosa* and degenerative and necrotic damage of Brunner’s glands are observed (Fig. 2D and 2E).

**Effects of NSAIDs on the number of PAS-positive goblet cells in the duodenal villi.** The effect of various COX inhibitors on the distribution of PAS-positive cells in the duodenal mucosa was examined in the same cats used for the ulcer experiments. In the cat duodenum, 2 types of mucus-containing cells (goblet cells and Brunner’s gland cells) were observed. Pretreatment with SC-560 and IND decreased the number of PAS-positive goblet cells in the villi, but celecoxib did not (Fig. 3A). The number of PAS-positive goblet cells in the villi in control cats was 5.46 ± 0.35/100 μm villus (n=4). Pretreatment with SC-560 and IND significantly (P<0.01 vs. control) decreased the number of goblet cells, i.e., 1.93 ± 0.22 and 2.71 ± 0.13/100 μm villus (n=4), respectively (Fig. 3B). Celecoxib had no effect on the number of goblet cells, i.e., 4.49 ± 0.62/100 μm villus (n=4).

**Effect of feeding and cimetidine on expression of COX isoforms and PGE₂ levels in the duodenum.** Expression of both isoforms of COX protein was observed in fasted cats, and their
expression was markedly increased in fed cats; such an increase was prevented by pretreatment with cimetidine (Fig. 4A). The relative intensity values of COX-1 expression in cats that were fasted, fed, or fed and pretreated with cimetidine were 1.0 ± 0.1, 2.4 ± 0.6 (P<0.05 vs. fasted cats) and 1.3 ± 0.1 fold (n=4), respectively (Fig. 4B). Those of COX-2 expression were 1.0 ± 0.4, 4.7 ± 1.4 (P<0.05 vs. fasted cats) and 1.1 ± 0.4 (P<0.05 vs. fed cats) fold (n=4), respectively (Fig. 4B).

PGE2 levels in the duodenum are shown in Fig. 4C. The level in fasted cats was 179.5 ± 8.1, pg/mg protein (n=4), and was markedly higher in fed cats (289.9 ± 19.0 pg/mg protein, n=4, P<0.01 vs. fasted cats). The PGE2 level was markedly decreased by pretreatment with cimetidine (181.2 ± 27.5 pg/mg protein, n=4, P<0.01 vs. fed cats).

**Localization of COX-1 and COX-2 in the duodenum.** As shown in Fig. 5, COX-1 or COX-2 immunoreactivity (IR) was recognized in various areas and cells in the duodenum of fed cats. COX-1-IR was observed markedly in goblet cells, Brunner’s gland cells and ganglion cells in both Meissner’s and Auerbach’s plexus; and moderately in smooth muscle cells, arterioles and villous columnar cells (Fig. 5B). In most of the positive cells, except for goblet cells, COX-1-IR was observed predominantly in the cytoplasm and nuclear areas. In contrast, COX-2-IR was observed in cytoplasm of endothelial cells of capillaries and venules, and basal granulated cells in both the villi and crypts (Fig. 5C). As in fed cats, COX-1-IR and COX-2-IR were observed in
fasted cats, although the staining was weak (not shown).
Discussion

Although it is well known that NSAIDs cause ulcers in the duodenum of humans, the role of COX isoforms in the pathogenesis of duodenal ulcers has not been well studied, probably due to the lack of suitable experimental animal models for duodenal ulcers induced by NSAIDs. In the previous study we reported that IND produced marked ulcers in the duodenum of cats (Satoh et al., 2009), suggesting that cats would be a good animal model to elucidate the role of COX isoforms in the pathogenesis of duodenal ulcers induced by NSAIDs.

It has been reported that NSAID-induced gastric and small intestinal lesions in rats and mice require simultaneous inhibition of both COX-1 and COX-2 (Wallace et al., 2000; Gretzer et al., 2001; Tanaka et al., 2001, 2002a; Sighthorsson et al., 2002). In the present study we examined the effect of selective COX-1 inhibitors (SC-560 and ketorolac) and COX-2 inhibitors (celecoxib and meloxicam) on the GI mucosa in cats, and found that all of these drugs alone, unexpectedly, produced marked lesions in the duodenum, though they did not produce obvious lesions in the small intestine. However, co-administration of low doses of SC-560 and celecoxib produced marked lesions in the small intestine as well as nonselective COX inhibitor IND. These results support the above notion that simultaneous inhibition of both COX-1 and COX-2 is necessary for the induction of small intestinal lesions by NSAIDs, and suggest that the role of COX isoforms in
the formation of duodenal ulcers is different from that in the small intestine. Also suggested is
that inhibition of either COX-1 or COX-2 alone will be sufficient to produce ulcers in the
duodenum in cats.

Gretzer et al. (2001) found that inhibition of both COX isoforms is necessary to cause lesions
in normal rat stomach, while COX-1 inhibition produces lesions in the stomach in the presence of
0.2 N HCl, indicating that COX-1 inhibition can produce gastric lesions in the presence of
noxious substances. In cats, as well as in humans, the duodenal mucosa is periodically exposed
to noxious substances such as strong gastric acid and bile acids after a meal. This suggests that
the duodenal mucosa of cats is sensitive to gastric acid or a rapid post-prandial response exists,
and has a specific protective mechanism against the noxious effects of gastric acid. We
demonstrated that both the expression of COX isoforms and the levels of PGE2 in the duodenum
were increased after a meal and that the effect was suppressed by pretreatment with cimetidine,
suggesting that the influx of gastric acid into the duodenum stimulates the local expression of
COX isoforms and that the increase in COX isoforms contributes to the protection of mucosa
through the enhanced PG synthesis during the post-prandial period. We previously reported that
the formation of IND-induced duodenal lesions in cats was markedly prevented by pretreatment
with cimetidine or misoprostol (Satoh et al., 2009, 2010). This finding was consistent with the
present results. Furthermore, in the present study, the duodenal lesions induced by SC-560 and
celecoxib were significantly increased when the drugs were administered 1 h before a feeding. Therefore, up-regulation of both COX-1 and COX-2 by luminal acid exposure after feeding or by other stimuli during the post-prandial period may enhance duodenal mucosal protection, possibly through different mechanisms, since our results showed the differences in the distribution of COX-1 and COX-2 positive cells. Inhibition of either COX-1 or COX-2 before or after feeding decreased the protective activity of the mucosa, and thereby caused lesions in the duodenum, though we did not examine the effect of each COX inhibitor on the increase of PGE2 levels induced by feeding.

To elucidate the mechanism of lesion formation by selective COX inhibitors we examined the effect of COX inhibitors on the distribution of mucus, an important factor in the protection of duodenal mucosa (Akiba et al., 2000, 2001). We found that the number of PAS-positive goblet cells in the villi was significantly decreased by SC-560 and IND, but not by celecoxib, suggesting that production of mucus in goblet cells depends on PGs produced by COX-1 but not by COX-2. We previously reported that SC-560, but not rofecoxib, a selective COX-2 inhibitor, increased intestinal motility in rats (Tanaka et al., 2001). Wallace et al. (2000) found that SC-560, but not celecoxib, decreased mucosal blood flow in rat stomach. Furthermore, COX-1 is involved in the local regulation of acid-induced duodenal HCO3⁻ secretion in rats (Takeuchi et al., 2002b). In the present study, COX-1-IR was observed markedly in goblet cells, Brunner’s gland cells and
ganglion cells in both Meissner’s and Auerbach’s plexus; and moderately in smooth muscle cells, arterioles and villous columnar cells. Taken together, these findings suggest that inhibition of COX-1 causes lesions in the duodenum by increasing motility and decreasing mucus production, mucosal blood flow, and HCO₃⁻ secretion in the presence of noxious substances such as gastric acid and bile acids during the post-prandial period.

It has been reported that the ulcerogenic activity of selective COX-2 inhibitors on the GI tract is weaker than that of non-selective COX inhibitors both in rats (Futaki et al., 1993; Seibert et al., 1994; Laudanno et al., 2001) and humans (Laine et al., 1999; Simon et al., 1999; Silverstein et al., 2000; Bombardier et al., 2000). However, Cheung et al. (2010) recently reported that the incidence of duodenal ulcer by the administration of celecoxib (100 mg, BID) and diclofenac (50 mg, BID), which was examined in 880 patients with osteoarthritis and rheumatoid arthritis, did not differ significantly between those taking celecoxib and diclofenac (2.3% vs. 1.5%). Thus it was shown that the selective COX-2 inhibitor celecoxib produces duodenal ulcers to the same degree as the non-selective COX inhibitor diclofenac in humans. In the present study the selective COX-2 inhibitors tested, as well as IND, invariably produced severe lesions (ulcers) in the duodenum. Laudanno et al. (2001) reported that the selective COX-2 inhibitors, rofecoxib and celecoxib, did not produce lesions in the GI mucosa in normal rats, but that the drugs aggravated GI lesions induced by various ulcerogenic stimuli such as water-immersion stress, IND, and
cysteamine, and delayed the healing of gastric ulcers induced by acetic acid. Gretzer et al. (1998) reported that selective COX-2 inhibitors negated the protective activity of the stomach caused by a mild irritant (20% ethanol) in rats. Wallace et al. (2000) found that celecoxib, but not SC-560, accelerated the adhesion of leukocytes to intestinal microvessels in rats, suggesting that leukocyte adhesion induced by COX-2 inhibition contributes to NSAID-induced gastric lesions. This is consistent with the present findings that COX-2-IR, but not COX-1-IR, was observed in endothelial cells of capillaries and venules in the duodenum. Together, these findings suggest that inhibition of COX-2 decreases the protective and healing activity of the mucosa, where COX-2 is adaptively or protectively induced by various stimuli, and thereby causes or aggravates lesions in the GI tract under some pathophysiological conditions.

We found that both COX isoforms were present in the duodenum and that the expressions were up-regulated by feeding, that COX-1 and COX-2 positive responses were observed in different cells, and that inhibition of either COX-1 or COX-2 alone caused ulcers in the duodenum, suggesting that COX-1 and COX-2 differently protect the duodenal mucosa in cats. Additional studies to elucidate the role of COX isoforms in the protection of GI mucosa under various pathophysiological conditions could provide useful information regarding NSAID safety.

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**Authorship Contributions.**

- Participated in research design: Satoh, Takeuchi
- Conducted experiments: Satoh, Amagase, Ebara
- Performed data analysis: Satoh, Amagase, Ebara
- Wrote or contributed to the writing of the manuscript: Satoh, Akiba, Takeuchi
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Legends for Figures

Fig. 1 Effects of NSAIDs on the GI mucosa of the cat. NSAID-induced duodenal (A) and small intestinal (B) lesions induced by various NSAIDs. NSAIDs were administered p.o. or s.c. (MEL) just after the morning meal once daily for 3 days. SC (SC-560), KET (ketorolac), CEL (celecoxib), MEL (meloxicam). ( ): dose (mg/kg) of NSAIDs. Data show the mean ± SEM values (n=4). **: P<0.01 vs. SC-560 (3) or CEL (10) alone (Student’s t-test).

Fig. 2 Duodenal lesions induced by selective COX inhibitors. Either SC-560 (10 mg/kg) or celecoxib (10 mg/kg) was administered p.o. just after the morning meal once daily for 3 days. Macroscopic observation of duodenal lesions of cats given SC-560 (A) or celecoxib (B). Microscopic observation of normal duodenum (C), duodenal lesions induced by SC-560 (D) or celecoxib (E) (H.E. staining).

Fig. 3 Effect of NSAIDs on the distribution of PAS-positive goblet cells in the duodenal mucosa (PAS staining). SC-560 (10 mg/kg), celecoxib (CEL, 10 mg/kg) and IND (3 mg/kg) were administered p.o. once daily for 3 days. (A) Typical photographs of duodenal mucosa in each group. (B) Effect of NSAIDs on the number of PAS-positive goblet cells in villi. Data represent
Fig. 4 Effect of feeding and cimetidine on expression of COX isoforms and PGE2 levels in the duodenum. Typical photographs of expression of COX isoforms (A), relative intensity values of COX-1 and COX-2 expression (B) and PGE2 levels (C) in each group. Duodenal samples were obtained before (Fasted) and 0.5 h after a 1-h feeding of diet (Fed) after a 16-h fast. Cimetidine (40 mg/kg, p.o.) was administered 1 h before feeding, and duodenal samples were obtained 0.5 h after a 1-h feeding period (CIM + Fed). Data represent mean ± SEM values (n=4). *: p<0.05 **: P<0.01 vs. Fasted group, #: p<0.05, ##: p<0.01 vs. Fed group, (Student’s t-test)

Fig. 5 Localization of COX-1 and COX-2 isoforms in the duodenum in fed cats (immunohistochemical observation). Positive responses to COX antibodies are shown by brown color, and specific cells are shown by arrowhead. (A) Localization of COX isoforms in the duodenum; (1) COX-1, (2) COX-2, (3) Negative control (N.C.). LMM: lamina muscularis mucosa, SM: submucosa, BG: Brunner’s glands, TM: tunica muscularis. (B) Localization of COX-1; (1) Villous (Co: villous columnar cells, Go: goblet cells), (2) Brunner’s gland (Mu: mucus cells, Fb: fibroblast, Mc: mesenchymal cells, En: endothelial cells), (3) arterioles (Sm: smooth muscle cells), (4) Meissner’s plexus (Ga: ganglion cells), (5) circular (CM) and longitudinal (LM) smooth
muscle layers, Auerbach's plexus (AP, Sc: Schwann cells), and arteriole (AR). (C) Localization of COX-2; (1) villous (Bg: basal granulated cells), (2) crypt, (3) Brunner's gland (Ca: capillaries), (4) circular smooth muscle layer, (5) venules in smooth muscle layer
Table 1. Comparison of the ulcerogenic effect of selective COX inhibitors given before or after feeding of diet. COX inhibitors were administered orally 1 h before or just after the morning meal once a day for 3 days. Lesions were examined 24 h after the final dose of the COX inhibitors.

Data represent mean ± SEM.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Timing of drug administration</th>
<th>No. of cats</th>
<th>Lesion index in the duodenum (cm²)</th>
<th>Lesion index in the small intestine (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC-560 (3)</td>
<td>After feeding</td>
<td>4</td>
<td>0.4 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>SC-560 (3)</td>
<td>Before feeding</td>
<td>4</td>
<td>1.2 ± 0.3*</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Celecoxib (10)</td>
<td>After feeding</td>
<td>4</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>Celecoxib (10)</td>
<td>Before feeding</td>
<td>4</td>
<td>1.3 ± 0.2*</td>
<td>0.8 ± 0.3</td>
</tr>
</tbody>
</table>

*: p<0.05 vs. “after feeding”, SC-560 or Celecoxib, respectively. (Student’s t-test)
(Fig. 2)
A

Control  SC-560  CEL  IND

B

No. of PAS positive goblet cells/100 μm villus

Control  SC-560  CEL  IND

(Fig. 3)
(Fig. 4)
(Fig. 5)