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CINACALCET, A CALCIMIMETIC, PREVENTS NONSTEROIDAL ANTIINFLAMMATORY DRUG-INDUCED SMALL INTESTINAL DAMAGE IN RATS

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The extracellular calcium-sensing receptor (CaSR), a G protein-coupled cell receptor cloned from bovine parathyroid, has been demonstrated to play a regulatory role in various functions of the gastrointestinal tract. In the present study, we examined the effect of cinacalcet, a drug that acts as a calcimimetic through the allosteric activation of CaSR, on the loxoprofen-induced small intestinal lesions and investigated the mechanisms involved in the protective action. Male Sprague-Dawley rats were used without fasting. The animals were administered loxoprofen p.o. and euthanized 24 hours later and the intestinal mucosa was examined for lesions. Cinacalcet was given p.o. twice, 30 min before and 6 h after loxoprofen. Loxoprofen caused hemorrhagic lesions in the small intestine, accompanied by the upregulation of enterobacterial invasion, myeloperoxidase (MPO) activity and inducible nitric oxide synthase (iNOS)/tumor necrosis factor α (TNF- α) expression as well as the downregulation of Muc2 expression. Prior administration of cinacalcet dose-dependently and significantly reduced the severity of these lesions in response to loxoprofen, with concomitant suppression of the changes in bacterial invasion, iNOS/TNF- α as well as Muc2 expression, and myeloperoxidase activity. Cinacalcet also significantly reversed a decrease in mucus secretion and fluid secretion in the small intestine caused by loxoprofen, but had no effect on the intestinal hypermotility or prostaglandin E₂ deficiency caused by loxoprofen. These results suggest that cinacalcet protects the small intestine against loxoprofen-induced damage, and this effect may be functionally associated with an increase in fluid secretion and a reversal of downregulation of Muc2 expression caused by loxoprofen, resulting in suppression of bacterial invasion and iNOS/TNF- α expression, the major pathogenic events in nonsteroidal antiinflammatory drugs -induced small intestinal ulceration.

Key words: *intestinal damage, calcium-sensing receptor, cinacalcet, loxoprofen-induced enteropathy, inducible nitric oxide synthase, myeloperoxidase, tumor necrosis factor-alpha*

INTRODUCTION

Nonsteroidal antiinflammatory drugs (NSAIDs) cause intestinal ulceration in humans and laboratory animals after short-term and long-term administration (1, 2). In particular, recent clinical studies using capsule endoscopes or double-balloon endoscopes confirmed that NSAIDs damage the small intestine in patients at a higher incidence than previously thought (3-5). Although several factors are involved in the pathogenesis of these lesions, including bile acid, bacterial flora and nitric oxide (NO) (6-8), a deficiency of endogenous prostaglandins (PGs) due to the inhibition of cyclooxygenase (COX) is of prime importance to the ulcerogenic response to NSAIDs. In addition, it should be noted 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 (7, 9). At present, no satisfactory means for the prevention and treatment of these lesions are currently available, except for the use of PG analogs (5). This situation has been much intensified by the recent findings that antisecretory drugs, such as proton pump inhibitors

and histamine H₂ receptor antagonists, are ineffective against NSAID-induced small intestinal damage (3, 6) and even worsen the severity of these lesions (10, 11), although they prevent the gastric ulcerogenic response to NSAIDs (11). Thus, the identification of effective therapies for the treatment of NSAID-induced small intestinal lesions remains an urgent priority.

The extracellular calcium sensing receptor (CaSR), which is a G protein-coupled receptor cloned from the bovine parathyroid gland (12), is known for its pivotal role in serum calcium homeostasis (13). Interestingly, the expression of CaSR has been observed throughout the entire gastrointestinal tract (14, 15). In the mammalian stomach, CaSR has been identified at both the apical and the basolateral membranes of G cells (16, 17) and mucous-secreting cells (18), whereas in parietal cells the receptor has been detected only at the basolateral membranes (19). In the small intestine, these receptors are expressed on both the apical and the basolateral membranes of villus cells (20). Recently, many studies have also demonstrated that CaSR regulates various functions in the gastrointestinal tract, such as gastric acid secretion (21), colonic fluid transport (22), and

intestinal epithelial cell growth and differentiation (14, 15, 23). However, it remains unknown whether CaSR plays a role in the pathogenesis of NSAID-induced small intestinal damage. NSAIDs decrease mucus/fluid secretions in the small intestine, the events involved in the pathogenesis of NSAID-induced intestinal damage (24). Since CaSR is reportedly known to modulate intestinal fluid secretion (22), it is possible that the activation of CaSR exhibits a prophylactic effect against the intestinal ulcerogenic response to NSAIDs.

In the present study, we examined the effect of cinacalcet, a drug acting as a calcimimetic by activating CaSR in an allosteric manner, on the development of small intestinal lesions caused by loxoprofen, a NSAID frequently used in Asian countries, and investigated the mechanisms involved in the protective action. Since γ -glutamyl peptides are known to be CaSR agonists (25), we also examined the effect of γ -glutamine-valine-glycin (γ -Glu-Val-Gly) on this model of intestinal lesions to confirm the role of CaSR in the pathogenesis of NSAID-induced enteropathy.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200–260 g; Nippon Charles River, Shizuoka, Japan) were acclimated to standard laboratory conditions (12:12 h light-dark cycle, temperature of $22\pm 1^\circ\text{C}$). Experiments were carried out using four to six non-fasted animals in a conscious state, unless otherwise specified. All experimental procedures involving animals were approved by the Experimental Animal Research Committee of the Kyoto Pharmaceutical University.

Induction of small intestinal damage by loxoprofen

Animals were administered loxoprofen (60 mg/kg) p.o. and euthanized 24 hours later under deep ether anesthesia. The small intestine was excised, the tissue walls fixed with 2% formalin for 10 min, and an opening made along the anti-mesenteric attachment. The area of macroscopically visible damage (mm^2) was measured under a dissecting microscope with square grids ($\times 10$), summed per tissue, and used as a lesion score. To highlight hemorrhagic lesions, a 1% Evans blue solution was administered i.v. in a volume of 1 mL/animals 30 min before killing. The person measuring the lesions did not know the treatment given to the animals. CaSR agonists, such as cinacalcet (1–100 mg/kg) and γ -Glu-Val-Gly (10–1000 mg/kg), were given p.o. twice 30 min before and 6 h after the administration of loxoprofen.

In some cases, the intestinal mucosa was examined with a light microscope following the administration of loxoprofen (60 mg/kg) with or without cinacalcet (10 mg/kg) or γ -Glu-Val-Gly (100 mg/kg). The animals were euthanized under deep diethyl ether anesthesia 24 hours after the loxoprofen treatment, and the small intestines excised. The tissue samples were then immersed in 10% neutralized formalin, embedded in paraffin, sectioned at 5 μm , and stained with hematoxylin and eosin (H&E).

Determination of myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured according to a modified method of Krawisz *et al.* (26). The animals were given loxoprofen (60 mg/kg) or cinacalcet (10 mg/kg) p.o., either alone or in combination, and in the combined administration, cinacalcet was given 30 min before loxoprofen. Six hours after the administration of loxoprofen or cinacalcet, the animals were euthanized under deep diethyl ether anesthesia, and the small intestines were removed. After a rinse with cold

saline, the whole of the intestine was weighed and homogenized in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HTAB, pH 6.0; Sigma). The homogenized samples were subjected to freeze-thawing three times and centrifuged at 2000 g for 10 min at 4°C . The MPO activity was determined by adding 5 μL of the supernatant to 95 μL of 10 mM phosphate buffer (pH 6.0) and 50 μL of 1.5 M *o*-dianisidine HCl (Sigma) containing 0.0005% w/v hydrogen peroxide. The changes in absorbance at 450 nm of each sample were recorded on a microplate reader (VERSAmax, Molecular Device, Sunnyvale, CA). Sample protein content was estimated by spectro-photometric assay (protein assay kit; Pierce, Rockford, IL, USA), and the MPO activity was obtained from the slope of the reaction curve, according to the following equation; Specific activity ($\mu\text{mol H}_2\text{O}_2/\text{min}/\text{mg protein}$) = $(\text{OD}/\text{min})/\text{OD}/\mu\text{mol H}_2\text{O}_2 \times \text{mg protein}$.

Determination of enterobacterial counts

Enterobacteria were enumerated according to a method described by Deitch *et al.* (27). Six hours after loxoprofen treatment (60 mg/kg, p.o.), the animals were euthanized under deep ether anesthesia, and the small intestines were removed. After each intestine was rinsed with sterile saline, the mucosa was scraped with glass slides, weighed, and homogenized in 1 mL of sterile phosphate-buffered saline (PBS) per 100 mg of wet tissue. Aliquots of the homogenate were placed on blood agar and Gifuco anaerobic medium agar (Nissui, Tokyo, Japan). Blood agar plates were incubated at 37°C for 24 hours under aerobic conditions (BBL Gas Pack Pouch Anaerobic System; BD Biosciences, San Jose, CA). Plates containing 10 to 300 colony-forming units (CFU) were examined for numbers of enterobacteria, and the data were expressed as log CFU per gram of tissue. Cinacalcet (10 mg/kg) was given p.o. 30 min before the administration of loxoprofen.

Determination of inducible nitric oxide synthase, tumor necrosis factor- α and Muc2 mRNA expression

The expression of Muc2, inducible nitric oxide synthase (iNOS) and tumor necrosis factor α (TNF- α) mRNA in the small intestinal mucosa was measured by reverse transcriptional polymerase chain reaction (RT-PCR). The animals were given loxoprofen (60 mg/kg) or cinacalcet (10 mg/kg) p.o., either alone or in combination, and in the combined administration, cinacalcet was given 30 min before loxoprofen. They were euthanized under deep diethyl ether anesthesia 3 or 6 hours later for determination of Muc2 or iNOS/TNF- α mRNAs, respectively, and the small intestines were removed and stored at -80°C prior to use. In the combined administration, cinacalcet was given 30 min before loxoprofen. Total RNA was extracted from tissue samples using Sepasol RNA I (Nacalai Tesque, Kyoto, Japan). The total RNA was reverse-transcribed with a first strand cDNA synthesis kit (ReverTra Ace alpha, TOYOBO, Osaka, Japan). The sequences of the sense and antisense primers for rat iNOS, TNF- α , Muc2 and GAPDH and each product size are shown in Table 1. An aliquot of the RT reaction product served as a template in 35 cycles of PCR with 0.5 min of denaturation at 95°C and 1 min of extension at 68°C using the Advantage 2 polymerase mixture (CLONTECH, Mountain View, CA) in a thermal cycler (PC-806, ASTEC, Fukuoka, Japan). A portion of the PCR mixture was electrophoresed in 1.5% agarose gel in Tris-acetic acid-EDTA buffer (40 mM Tris, 20 mM acetic acid, and 2 mM EDTA; pH 8.1), and the gel was stained with ethidium bromide and photographed (Bio Doc-It Imaging System; UVP, Upland, CA, USA). Images were analyzed with the Image J (version 1.39), and the

semiquantitative measurement of mRNA expression was presented as a ratio compared with GAPDH.

Determination of mucus secretion

The amount of mucus secreted in the small intestine was determined by periodic acid-Schiff (PAS) staining. The animals were given loxoprofen (60 mg/kg) or cinacalcet (10 mg/kg) p.o., either alone or in combination, and in the combined administration, cinacalcet was given 30 min before loxoprofen. Three hours after the administration of loxoprofen or cinacalcet, the animals were euthanized under deep diethyl ether anesthesia, and the small intestines were removed. The removed tissues were fixed in Carnoy's fluid (ethanol: acetic acid: chloroform = 6: 1:3) for 24 hours, embedded in paraffin, and sectioned at a thickness of 8 μ m. PAS staining was performed according to the conventional method.

Determination of enteropooling

The enteropooling assay was performed according to the method described by Robert *et al.* (28). Animals were deprived of food for 24 hours but allowed free access to tap water until 1 hour before the experiment. The animals were given loxoprofen (60 mg/kg) or cinacalcet (10 mg/kg) p.o., either alone or in combination, and in the combined administration, cinacalcet was given 30 min before loxoprofen. Three hours after the administration of loxoprofen or cinacalcet, the animals were euthanized under deep diethyl ether anesthesia, and the abdomen was opened, and both the pylorus and the end of the ileum were clamped immediately. Then, the fluid accumulated in the lumen of the small intestine for 3 hours was collected in a graduated tube and centrifuged for 10 min at 3000 rpm, and volume was measured to the nearest 0.01 ml.

Determination of intestinal motility

Intestinal motility was measured according to a modified version (29) of the method originally reported by Calignano *et al.* (30). In brief, rats fasted for 18 hours 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 saline-filled balloon made from silicone rubber with 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 5–10 mmHg. The preparation was allowed to rest for 30 min, and intestinal motility was monitored on a recorder (U-228; Tokai-irika, Tokyo, Japan) as changes in intraluminal pressure through a pressure transducer and polygraph recorder (Nihon Kodan, Ibaraki, Japan). Loxoprofen (60 mg/kg) was given i.d. after basal intestinal motility had well stabilized, and the motility was measured for 3 hours thereafter. Cinacalcet (10 mg/kg) was given i.d. 2 hours after loxoprofen treatment.

Determination of mucosal prostaglandin E₂ content

Levels of prostaglandin E₂ (PGE₂) in the small intestine were measured after administration of loxoprofen and cinacalcet, either alone or in combination. Animals were given loxoprofen (60 mg/kg) or cinacalcet (10 mg/kg) p.o., either alone or in combination, and in the combined administration, cinacalcet was given 30 min before loxoprofen. Six hours after the administration of loxoprofen or cinacalcet, the animals were euthanized under deep diethyl ether anesthesia, and the small intestines were excised, weighed, and placed in a tube containing 100% methanol plus 0.1 M indomethacin. Then, the tissues were

homogenized using a polytron homogenizer (IKA, Tokyo, Japan) and centrifuged at 12,000 g for 10 min at 4°C. After the supernatant of each sample had been evaporated with N₂ gas, the residue was dissolved in assay buffer and used for determination of PGE₂. The concentration of PGE₂ was measured using a PGE₂ enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Preparation of drugs

The drugs used were loxoprofen Na (Sigma-Aldrich, St. Louis, MO), cinacalcet, γ -Glu-Val-Gly (Ajinomoto Co., Kawasaki, Japan), indomethacin (Sigma Chemicals, St. Louis, MO), and urethane (Tokyo Kasei, Tokyo, Japan). Loxoprofen, cinacalcet, and γ -Glu-Val-Gly were suspended in a 0.5% hydroxypropylcellulose solution (Wako Pure Chemicals, Osaka, Japan). Urethane was dissolved in saline. All drugs were prepared immediately before use and administered orally (p.o.) or intraperitoneally (i.p.) in a volume of 0.5 mL/100 g body weight or intraduodenally (i.d.) in a volume of 0.25 mL/100 g body weight. Control animals received the vehicle alone.

Statistical analyses

Data are presented as the mean \pm S.E. for four to six rats per group. Statistical analyses were performed using a two-tailed unpaired *t*-test and Dunnett's multiple comparison test, and values of $P < 0.05$ were considered significant.

RESULTS

Effect of cinacalcet on loxoprofen-induced small intestinal damage

Normal rats showed any damage in the small intestinal mucosa. Orally administered loxoprofen (60 mg/kg) in normally fed rats produced multiple hemorrhagic lesions in the small intestine, mainly in the ileum, the lesion score being 213.2 ± 45.1 mm² (Fig. 1A). When the animals were pretreated with cinacalcet, the CaSR agonist, given orally at 1–100 mg/kg, the development of these intestinal lesions was prevented in a dose-dependent manner, and a significant effect was observed at 10 mg/kg or greater, the inhibition at 10 mg/kg being 74.3%. In particular, this agent at 100 mg/kg almost totally suppressed the occurrence of these lesions in response to loxoprofen; the lesion score was 9.8 ± 5.0 mm². Likewise, another CaSR agonist, γ -Glu-Val-Gly (10–1000 mg/kg, p.o.), also reduced the severity of the loxoprofen-induced small intestinal damage, in a dose-dependent manner, with a significant effect obtained at 100 mg/kg or greater. Cinacalcet at 100 mg/kg or γ -Glu-Val-Gly at 1000 mg/kg alone did not cause any alteration in the small intestinal mucosa (data not shown).

Histologically, the lesions produced by loxoprofen were deep in the mucosa, almost reaching the muscularis mucosae, while the severity of the damage was markedly lessened in rats pretreated with cinacalcet (10 mg/kg) or γ -Glu-Val-Gly (100 mg/kg) (Fig. 1B).

Effect of cinacalcet on various events induced in the small intestinal mucosa by loxoprofen treatment

It was found that cinacalcet prevented the occurrence of damage in the small intestine after loxoprofen treatment, and this effect at 10 mg/kg was very potent and reproducible. To further investigate the functional mechanisms responsible for the protective action of cinacalcet, we examined the effect of cinacalcet at 10 mg/kg on various events that are considered critical in the pathogenesis of NSAID-induced enteropathy (31, 32).

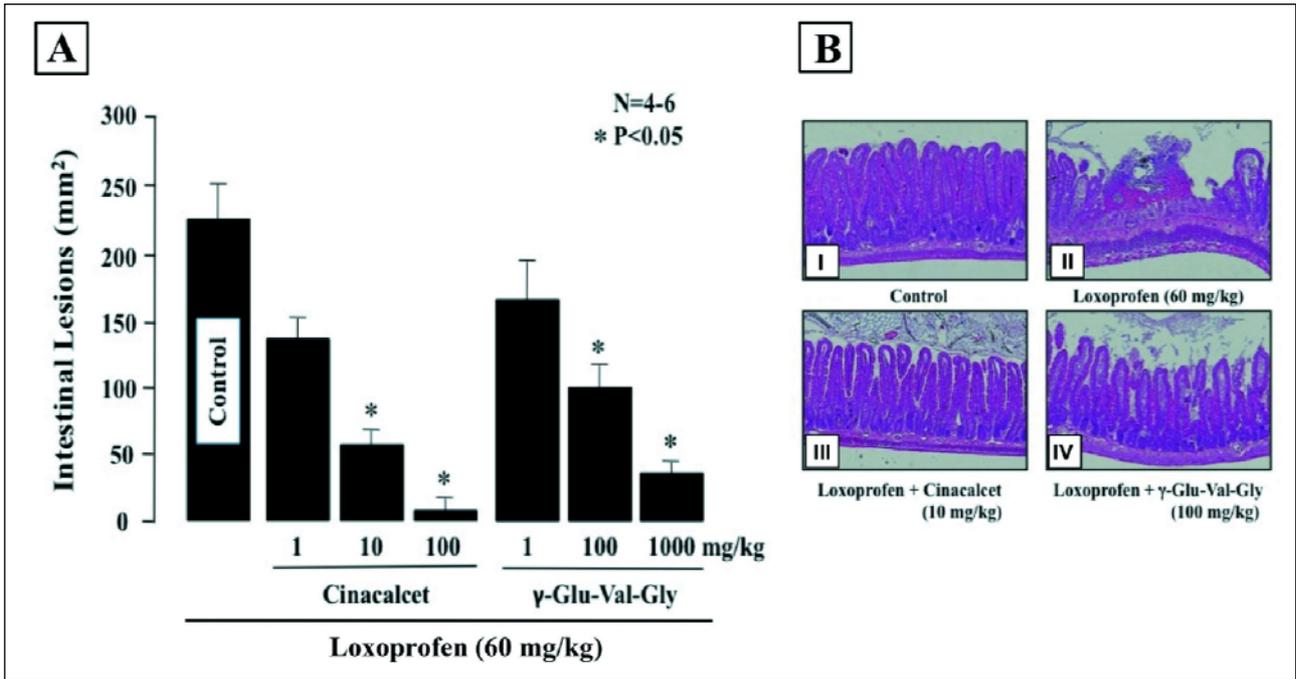


Fig. 1. (A) Effects of cinacalct and γ -Glu-Val-Gly on loxoprofen-induced small intestinal damage in rats. Animals were given loxoprofen (60 mg/kg) p.o. and euthanized 24 hours later. Cinacalct (1–100 mg/kg) or γ -Glu-Val-Gly (10–1000 mg/kg) was given p.o. twice, 30 min before and 6 hours after the administration of loxoprofen. Data are presented as the mean \pm S.E. for 4–6 rats. *Significant difference from control, at $P < 0.05$. (B) Microscopic observation of loxoprofen-induced small intestinal damage at 24 hours after loxoprofen treatment (H&E, $\times 40$). Note that loxoprofen produced severe lesions, extending to the muscularis mucosae, while the severity of the damage was markedly decreased in rats pretreated with cinacalct or γ -Glu-Val-Gly. Figures show; I: control; II: loxoprofen (60 mg/kg) alone; III: cinacalct (10 mg/kg) + loxoprofen; IV: γ -Glu-Val-Gly (100 mg/kg) + loxoprofen.

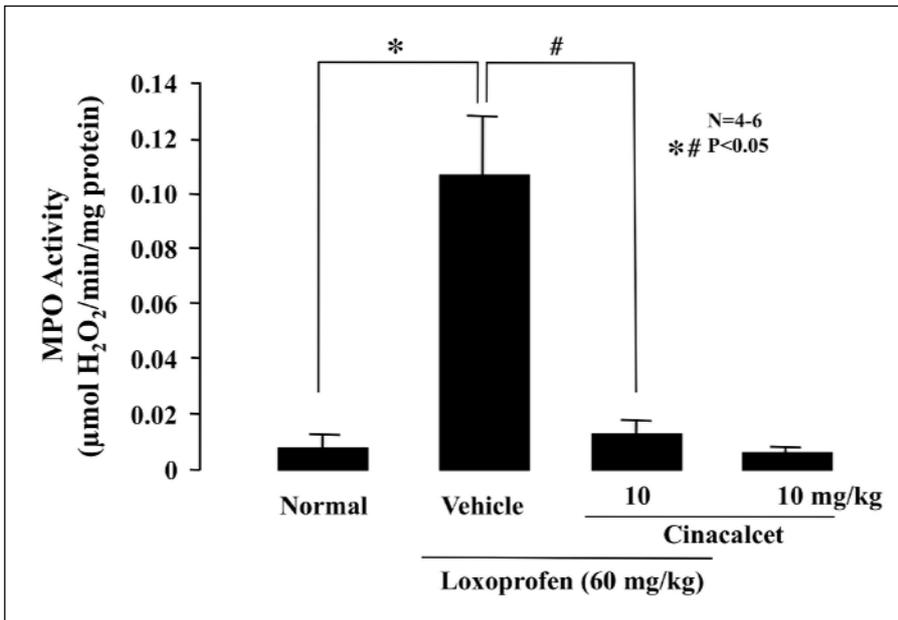


Fig. 2. Effect of cinacalct on MPO activity in the rat small intestine, with or without loxoprofen treatment. The animals were given cinacalct (10 mg/kg) or loxoprofen (60 mg/kg) p.o., either alone or in combination, and euthanized 6 hours later. In the combined administration, cinacalct was given 30 min before loxoprofen. Data are presented as the mean \pm S.E. for 4–6 rats. Significant difference at $P < 0.05$: * from normal; # from vehicle (loxoprofen alone).

1. Myeloperoxidase activity

The MPO activity in the normal intestinal mucosa was $0.007 \pm 0.006 \mu\text{mol H}_2\text{O}_2/\text{mg protein}$ and markedly elevated in response to loxoprofen (60 mg/kg), reaching $0.107 \pm 0.024 \mu\text{mol H}_2\text{O}_2/\text{min}/\text{mg protein}$ 6 hours later (Fig. 2). The elevated MPO activity after loxoprofen treatment was significantly suppressed by cinacalct (10 mg/kg), and the value decreased to $0.012 \pm 0.005 \mu\text{mol H}_2\text{O}_2/\text{min}/\text{mg protein}$, the inhibition being 88.8%.

Cinacalct alone had no effect on mucosal MPO activity in the small intestine.

2. Mucosal expression of inducible nitric oxide synthase and tumor necrosis factor- α mRNAs

GAPDH mRNA, the housekeeping gene, was clearly detectable in the small intestine of control rats and its expression remained unaffected by the administration of loxoprofen.

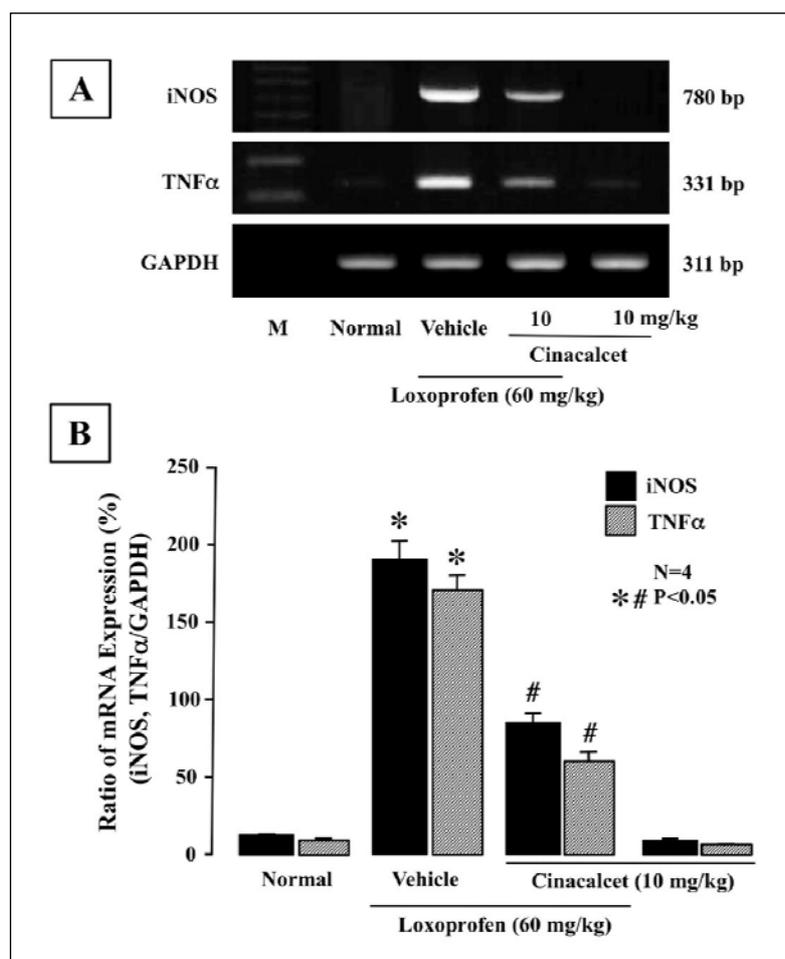


Fig. 3A. Effect of cinacalcet on the expressions of iNOS and TNF- α mRNA in the rat small intestine, with or without loxoprofen treatment. The animals were given cinacalcet (10 mg/kg) or loxoprofen (60 mg/kg) p.o., either alone or in combination, and euthanized 6 hours later for examination of the expression of iNOS and TNF- α mRNAs by RT-PCR. In the combined administration, cinacalcet was given 30 min before loxoprofen. M: Marker. Note that loxoprofen upregulated both iNOS and TNF- α expression, but these responses were apparently mitigated by prior administration of cinacalcet. In *Fig. 3B* densitometric quantitation was performed with image J software, and the results are expressed as the ratio of iNOS to GAPDH and TNF- α to GAPDH. Data are presented as the mean \pm S.E. for 4 rats. Significant difference at $P < 0.05$; * from normal; # from vehicle (loxoprofen alone).

Table 1. Sequences of sense and antisense primers for rat iNOS, TNF- α and Muc2.

Enzyme	Sequences	PCR Product
<u>iNOS</u>		780 bp
Sense	5'-CGGTTACAGTCTTGGTAAAAG-3'	
Antisense	5'-CAGGTGTTCCCAGGTAGGTAG-3'	
<u>TNFα</u>		331 bp
Sense	5'-GTCCAACAAGGAGGAGAAGTT-3'	
Antisense	5'-TCCTGGTATGAAGTGGCAAATC-3'	
<u>Muc2</u>		985 bp
Sense	5'-GCTCAATCTCAGAAGGCGACAG-3'	
Antisense	5'-GCTCAATCTCAGAAGGCGACAG-3'	
<u>GAPDH</u>		311 bp
Sense	5'-GAACGGGAAGCTCACTGGCATGGC-3'	
Antisense	5'-TGAGGTCCAC CACCTGTTGCTG-3'	

Expression of iNOS mRNA was not detected in the normal intestine, yet markedly upregulated in the mucosa when examined 6 hours after the administration of loxoprofen (60 mg/kg) (*Figs. 3A and 3B*). Pretreatment with cinacalcet (10 mg/kg) apparently suppressed the upregulation of iNOS expression caused by loxoprofen. Similarly, TNF- α mRNA expression was weak in the normal intestinal mucosa but markedly upregulated after the administration of loxoprofen. Again this upregulation was suppressed by the pretreatment with

cinacalcet. Cinacalcet alone did not cause any change in the expression of iNOS and TNF- α in the rat small intestine.

3. Enterobacterial invasion

Aerobic and anaerobic bacterial counts in the normal intestinal mucosa were 5.10 ± 0.10 and 5.87 ± 0.12 log CFU/g tissue, respectively (*Table 2*). Those following the administration of loxoprofen (60 mg/kg) were about 10 times greater after 6

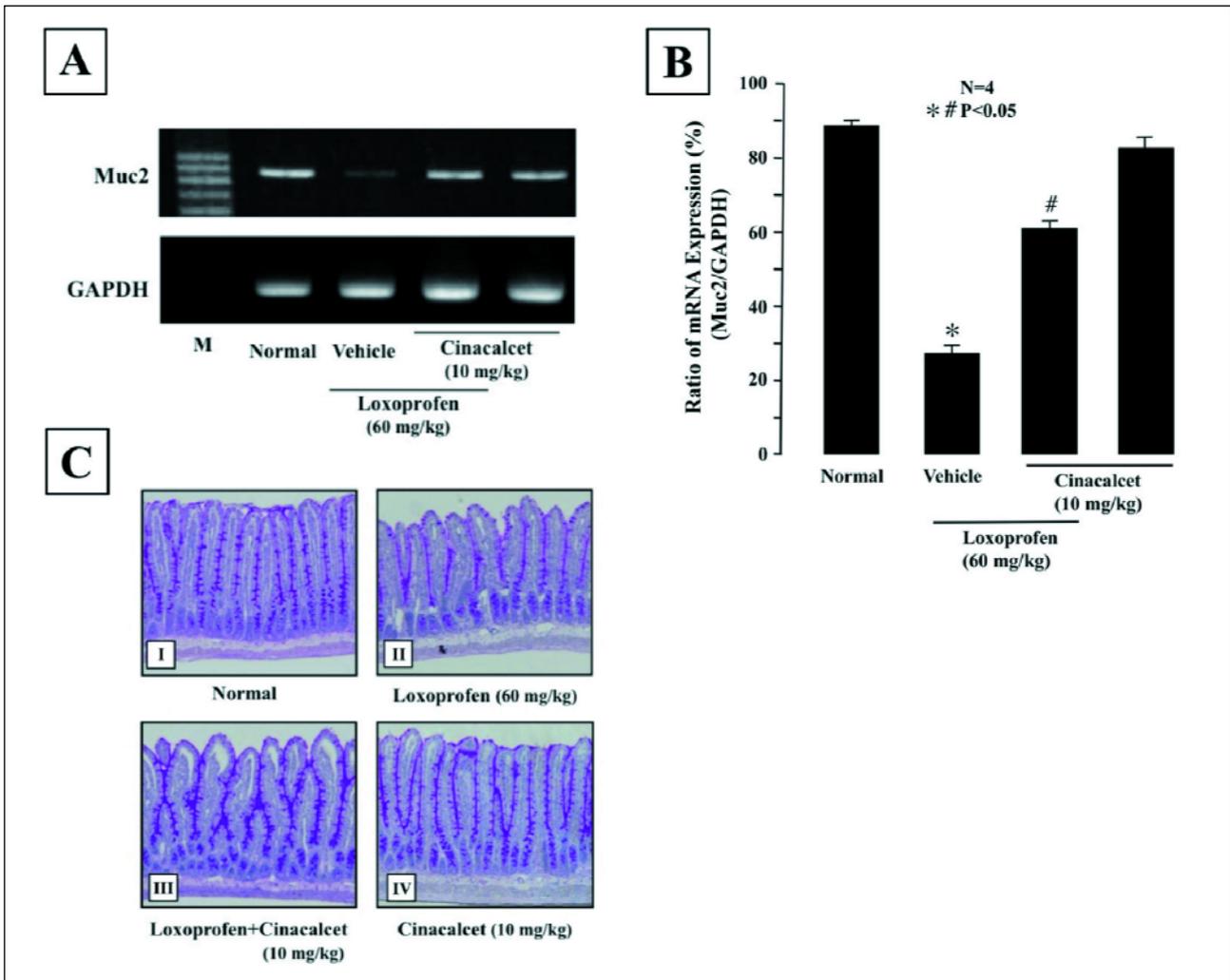


Fig. 4. Effect of cinacalcet on the expression of Muc2 mRNA and mucus secretion (PAS staining) in the rat small intestine, with or without loxoprofen treatment. The animals were given cinacalcet (10 mg/kg) or loxoprofen (60 mg/kg) p.o., either alone or in combination, and euthanized 3 hours later for examination of Muc2 expression and PAS staining. In the combined administration, cinacalcet was given p.o. 30 min before loxoprofen. (A) shows the expression of Muc2 mRNA by RT-PCR. M: Marker. In (B), densitometric quantitation was performed with image J software, and the results are expressed as the ratio of Muc2 to GAPDH. Data are presented as the mean \pm S.E. for 4 rats. Significant difference at $P < 0.05$; * from normal; # from vehicle (loxoprofen alone). (C) shows microscopic observations of the small intestinal mucosa ($\times 100$); normal (I), loxoprofen alone (II), cinacalcet plus loxoprofen (III), and cinacalcet alone (IV). Note that loxoprofen decreased mucus secretion, but this response was apparently prevented by prior administration of cinacalcet.

Table 2. Effect of cinacalcet on enterobacterial invasion in rat small intestine following loxoprofen treatment.

Treatment	N	Number of Bacteria (log CFU/g tissue)	
		Anaerobic	Aerobic
Normal	6	5.87 \pm 0.12	5.10 \pm 0.10
Loxoprofen	6	6.90 \pm 0.17*	6.27 \pm 0.11*
Cinacalcet + Loxoprofen	6	5.56 \pm 0.12#	5.29 \pm 0.14#

Rats were given loxoprofen (60 mg/kg) p.o., and the enterobacterial count was measured 6 hours later. Cinacalcet (10 mg/kg) was given p.o. 30 min before loxoprofen. Data are presented as the mean \pm S.E. for 6 rats. Significant difference at $P < 0.05$: * from normal; # from loxoprofen alone.

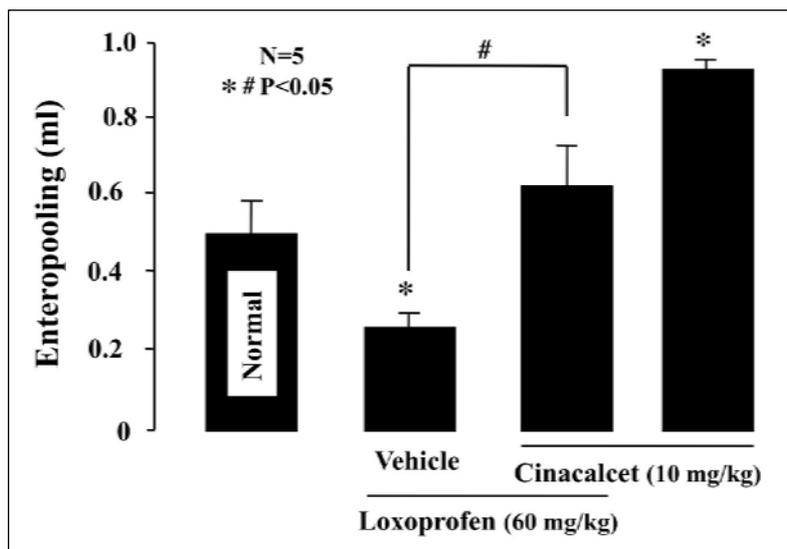


Fig. 5. Effect of cinacalcet on enteropooling (fluid secretion) in the rat small intestine, with or without loxoprofen treatment. The animals were given cinacalcet (10 mg/kg) or loxoprofen (60 mg/kg) p.o., either alone or in combination, and the amount of fluid accumulated in the small intestine was measured 3 hours later. In the combined administration, cinacalcet was given p.o. 30 min before loxoprofen. Data are presented as the mean \pm S.E. for 5 rats. Significant difference at $P < 0.05$: * from normal; # from vehicle (loxoprofen alone).

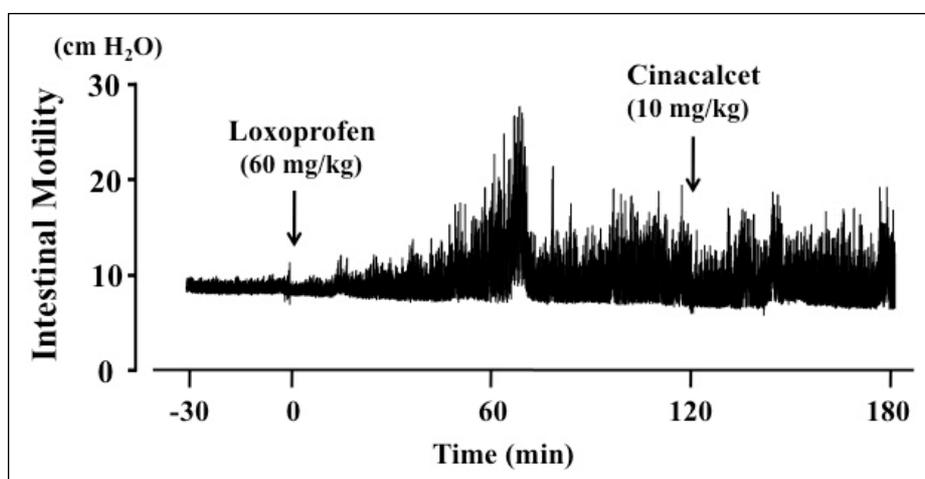


Fig. 6. Representative recordings showing the effect of cinacalcet on the enhanced small intestinal motility caused by loxoprofen in urethane-anesthetized rat. Loxoprofen (60 mg/kg) was given i.d., and small intestinal motility was recorded for 3 hours thereafter. Cinacalcet (10 mg/kg) was given i.d. 2 hours after the administration of loxoprofen. Note that cinacalcet had no effect on the intestinal hypermotility caused by loxoprofen.

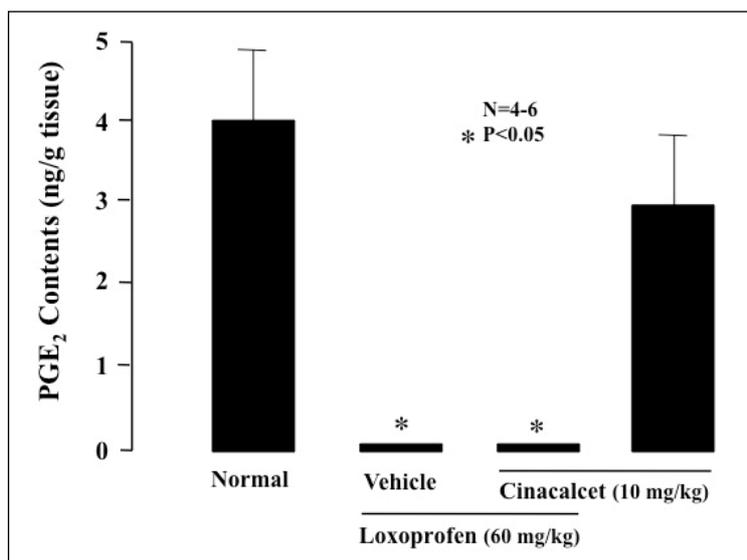


Fig. 7. Effect of cinacalcet on mucosal PGE₂ content in the rat small intestine, with or without loxoprofen treatment. The animals were given cinacalcet (10 mg/kg) or loxoprofen (60 mg/kg) p.o., either alone or in combination, and the mucosal PGE₂ content was measured 6 hours later. In the combined administration, cinacalcet was given p.o. 30 min before loxoprofen. Data are presented as the mean \pm S.E. for 6 rats. * Significant difference from normal, at $P < 0.05$.

hours, being 6.27 ± 0.11 and 6.90 ± 0.17 log CFU/g tissue, respectively. Enhanced invasion of enterobacteria after loxoprofen treatment was significantly prevented by pretreatment with cinacalcet (10 mg/kg), although this agent by itself had no effect on bacterial counts in the normal intestine (data not shown).

4. Mucosal expression of Muc2 mRNA and PAS-positive materials

Expression of Muc2 mRNA was detected in the normal intestinal mucosa, yet markedly downregulated after the

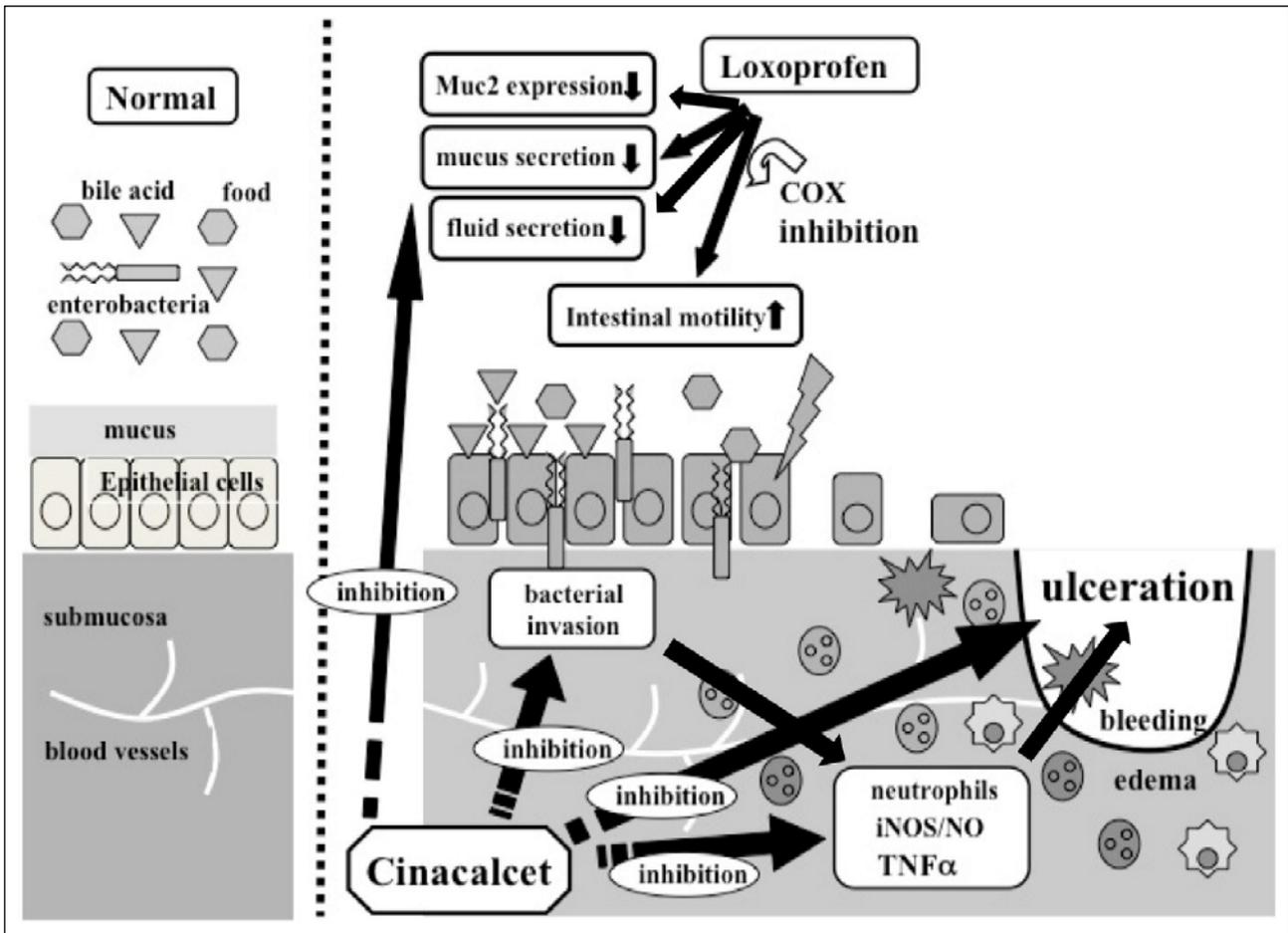


Fig. 8. Factors involved in the development of loxoprofen-induced small intestinal damage, and the influences of cinacalcet (the CaSR agonist) on these processes. Loxoprofen caused functional changes such as an increase in intestinal motility and a decrease in Muc2 expression/mucus secretion, followed by enterobacterial invasion in the mucosa. Endotoxin released from enterobacteria upregulated iNOS expression and NO production as well as inflammation, and by so doing resulted in damage to the small intestine. Cinacalcet increased mucus secretion through the upregulation of Muc2 expression and hampered the mucosal invasion of enterobacteria, and by so doing suppressed the upregulation of iNOS/TNF- α expressions and neutrophil migration, eventually damage in the small intestine.

administration of loxoprofen (60 mg/kg) when examined 3 hours later (Figs. 4A and 4B). The decrease in Muc2 expression caused by loxoprofen was all but totally reversed by pretreatment with cinacalcet (10 mg/kg). In the normal intestinal mucosa, PAS-positive materials were observed in the surface epithelial cells as well as the lamina propria mucosae along the intestinal gland (Fig. 4C-I). Loxoprofen apparently reduced the amount of PAS-positive materials in the small intestinal mucosa, particularly over the surface epithelial cells (Fig. 4C-II). When the animal was pretreated with cinacalcet before loxoprofen treatment, the decrease in PAS staining was prevented and the amount of PAS-positive materials in the mucosa was largely restored (Fig. 4B-III). Cinacalcet by itself did not cause apparent change in the expression of Muc2 mRNA and the amount of PAS-positive materials in the mucosa when compared to the control (Figs. 4A, 4B and 4C-IV).

5. Enteropooling

Control animals accumulated fluid in the small intestine, the amount being 0.50 ± 0.08 ml for 3 hours (Fig. 5). Oral administration of loxoprofen (60 mg/kg) significantly decreased the amount of fluid in the small intestine to

approximately 48% of the normal value. This effect of loxoprofen was significantly reversed by prior administration of cinacalcet (10 mg/kg), and the amount of fluid was restored, the value being 0.62 ± 0.11 ml. Furthermore, cinacalcet alone markedly increased the luminal accumulation of fluid, to about 1.8-fold that in normal rats.

6. Intestinal hypermotility

Under urethane anesthesia, intraduodenally administered loxoprofen (60 mg/kg) caused a marked enhancement in intestinal motility from 20–30 min after the administration, in terms of both the amplitude and frequency of contractions (Fig. 6). Cinacalcet (10 mg/kg), given 2 hours after loxoprofen, did not affect the intestinal hypermotility caused by loxoprofen.

7. Mucosal prostaglandin E_2 content

Loxoprofen (60 mg/kg) markedly decreased the mucosal PGE₂ content in the small intestine from 4.0 ± 0.9 ng/g tissue to 0.1 ng/g tissue within 6 hours. Cinacalcet (10 mg/kg) did not affect the mucosal PGE₂ content in the small intestine, in the absence or presence of loxoprofen (Fig. 7).

DISCUSSION

Extracellular CaSR functions as a key regulator of calcium homeostasis by detecting changes in ambient calcium concentrations. Recently, many papers have reported the expression of CaSR in various tissues of the gastrointestinal tract and physiological roles in these tissues (14-23). In the small intestine, the activation of CaSR modulates the secretion of fluid that may play an important role in maintaining mucosal homeostasis (22). Since NSAIDs decrease fluid secretions in the small intestine, the events involved in the pathogenesis of NSAID-induced intestinal damage (24), it is possible that the activation of CaSR enhances the mucosal defense in the small intestine against NSAIDs. Thus, we examined whether or not cinacalcet, a drug that acts as a calcimimetic through allosteric activation of CaSR, affords protection against NSAID-induced small intestinal damage in rats.

The present study showed that cinacalcet significantly prevented the development of small-intestinal lesions in response to loxoprofen, a NSAID frequently used in Asian countries including Japan (31). We previously reported that loxoprofen caused multiple hemorrhagic lesions in the rat small intestine, and the severity of these lesions observed at 60 mg/kg was all but equivalent to that of the lesions generated by indomethacin at 10 mg/kg (32). It was also known that the development of these lesions was accompanied by intestinal hypermotility, downregulation of Muc2 expression/mucus secretion and enterobacterial invasion as well as upregulation of iNOS expression, events that have been observed after the administration of indomethacin (7, 9, 29, 33).

Several factors have been implicated in the pathogenesis of NSAID-induced small intestinal ulceration, including bacterial flora, bile acid, and hypermotility, in addition to PG deficiency (7-9, 29, 34-36). The present study showed that mucosal PGE₂ content was markedly reduced after administration of loxoprofen, confirming a prostaglandin deficiency in the background for the intestinal ulcerogenic property (32). We previously found that a selective COX-1 inhibitor did not provoke any gross damage in the small intestine, because it up-regulated the expression of COX-2 mRNA/protein and the PGE₂ derived from COX-2 suppressed the deleterious events resulting from COX-1 inhibition (7). Since the activation of CaSR reportedly stimulated COX-2 expression and PGE₂ secretion in jaw cyst fibroblasts (37), it is possible that the protective action of cinacalcet in the small intestine may also be brought about by PGE₂ production due to COX-2 induction. However, it is unlikely that cinacalcet prevented the loxoprofen-induced intestinal damage by increasing levels of endogenous PGs, because this agent itself had no effect on basal PGE₂ production in the small intestine and did not affect the decreased PGE₂ content in the presence of loxoprofen. These findings suggest that cinacalcet may act downstream of the events resulting from the PG deficiency caused by loxoprofen and eventually prevents the development of small intestinal lesions.

Robert and Asano (36) reported that germ-free rats were resistant to indomethacin-induced intestinal damage, suggesting a key pathogenic role for enterobacteria in this model. Indeed, these intestinal lesions were prevented by pretreatment with antibiotics such as ampicillin (6, 7, 29, 33, 34). Boughton-Smith *et al.* (38) reported that bacterial endotoxin enhanced intestinal permeability through up-regulation of iNOS and overproduction of NO in the mucosa. This was further supported by the finding that indomethacin increased iNOS activity and NO production, preceding the onset of intestinal damage, and that aminoguanidine prevented the intestinal ulcerogenic response by suppressing NO production due to iNOS (27, 33, 39). Consistent with our recent observations (40), the present study confirmed that loxoprofen caused bacterial invasion in the mucosa,

followed by the upregulation of iNOS expression and MPO activity, similar to indomethacin, and these responses were suppressed by cinacalcet. These findings suggest that the protective effect of cinacalcet against loxoprofen-induced intestinal damage is functionally associated with the downregulation of iNOS expression and neutrophil recruitment resulting from the suppression of bacterial invasion. Zwolinska-Wcislo *et al.* (41) recently showed an importance of enterobacteria in the NSAID-induced delayed healing of the experimental colitis in rats and demonstrated that ampicillin was effective in the diminishing of the severity of colonic damage and accelerated the delayed healing of colonic damage (41). It is possible that cinacalcet may also be effective in accelerating the healing of NSAID-induced small intestinal damage by suppression of enterobacterial translocation.

The proinflammatory cytokine TNF- α is also capable of inducing iNOS expression in various cells. Bertrand *et al.* (42) demonstrated that NSAID induced local production of TNF- α in the small intestine and this event occurred before the elevation of NO production and MPO activity as well as lesion formation. Reuter and Wallace (43), however, reported that TNF- α does not play a critical role in NSAID-induced small intestinal injury, because inhibition of TNF- α release with thalidomide or immunoneutralization with a polyclonal antibody against TNF- α failed to afford any protection against indomethacin-induced enteropathy. In the present study, we confirmed the increased expression of TNF- α mRNA in the small intestine after loxoprofen treatment and found that cinacalcet apparently suppressed the increase. Thus, there is controversy over the involvement of TNF- α in the pathogenesis of NSAID-induced enteropathy, yet it is possible that the protective effect of cinacalcet in the present study is accounted for, at least partly, by suppression of inflammatory mediators such as TNF- α and iNOS after loxoprofen treatment. Kelly *et al.*, (44) reported that CaSR inhibited lipopolysaccharide-stimulated TNF signaling by modifying Cullin1 deneddylation in mouse peritoneal macrophages, RAW264.7 cells. We previously showed that an antibiotic ampicillin potently inhibited the up-regulation of iNOS as well as TNF- α following the administration of indomethacin, suggesting a relationship between the mucosal invasion of enterobacteria and the up-regulation of these cytokines (29, 33, 45). Thus, it is assumed that cinacalcet may inhibit TNF- α expression through at least two ways, such as suppression of enterobacterial invasion and modification of Wnt signaling.

The mechanism by which enterobacteria invade the mucosa remains unknown, yet previous studies suggest that a decrease in mucus secretion may contribute to this process after indomethacin treatment (24, 32, 46). Since mucus plays a crucial role in innate host defenses against intestinal pathogens and irritants, it is possible that a decrease in mucus secretion weakens the intestinal barrier, resulting in bacterial invasion. In the present study, we found that the amount of PAS-positive materials in the small intestine was markedly reduced after loxoprofen treatment, and cinacalcet restored the decrease in PAS-positive materials caused by loxoprofen. Consistent with previous findings (32, 47), loxoprofen decreased the expression of Muc2 mRNA in the small intestine and this response was totally attenuated by cinacalcet treatment. Muc2, an important mucin, plays a major role in the dimerization of secretory mucin, an essential step in the formation of gastrointestinal mucus-gels (48). It is assumed that cinacalcet caused a reversal of downregulation of Muc2 expression and mucus secretion caused by loxoprofen, thereby maintaining the mucus gel's thickness and hampering bacterial invasion following the administration of loxoprofen. Likewise, the secretion of intestinal fluid prevents bacterial invasion by washing out the invading bacteria (7, 9, 28, 49). Loxoprofen decreased the amount of fluid accumulated in

the small intestine, and this response was reversed by the prior administration of cinacalcet. We also found that cinacalcet itself markedly enhanced the accumulation of fluid in the small intestine, suggesting the stimulation of intestinal fluid secretion by the activation of CaSR.

Intestinal hypermotility is also considered to play a role in the pathogenesis of NSAID-induced small intestinal damage (29, 50). Abnormal hypermotility caused by NSAIDs may disrupt the unstirred mucus layer over the epithelium, leading to the expedition of enterobacterial invasion. Similar to other NSAIDs (29), loxoprofen enhanced intestinal motility, and this response occurred within 20–30 min after its administration, much sooner than the onset of other pathogenic events such as bacterial invasion, neutrophil activation and iNOS expression. Since the activation of CaSR raises intracellular Ca²⁺ concentrations by stimulating inositol triphosphate turn-over, it is assumed that cinacalcet exhibits a positive influence on intestinal motility. Unexpectedly, cinacalcet did not significantly affect the intestinal hypermotility caused by loxoprofen. At present, the reason why cinacalcet had no effect on smooth muscle contraction remains unknown.

In conclusion, we found that cinacalcet prevents loxoprofen-induced small intestinal damage in rats. This calcimimetic, by activating the CaSR, enhanced intestinal barrier function through a reversal of downregulation of Muc2 expression/mucus secretion and fluid secretion caused by loxoprofen, resulting in suppression of bacterial invasion and iNOS expression, and by so doing prevented the development of intestinal damage in response to loxoprofen (Fig. 8). It is assumed that CaSR agonists, such as cinacalcet, can be used as a prophylactic agent against NSAID-induced small intestinal damage.

Conflict of interests: None declared.

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