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SMALL INTESTINE

Prophylactic Effect of Irsogladine Maleate Against Indomethacin-Induced Small Intestinal Lesions in Rats

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Abstract The effect of irsogladine maleate, a widely used antiulcer drug in Japan, on indomethacin-induced small intestinal lesions was examined in rats. Animals without fasting were given indomethacin (10 mg/kg, s.c.) and sacrificed 24 h later. Irsogladine (1–10 mg/kg) or 16,16-dimethyl prostaglandin E_2 ($dmPGE_2$ 0.03 mg/kg) was given p.o. twice, 0.5 before and 6 h after indomethacin, while ampicillin (800 mg/kg) was given twice, 18 and 0.5 h before. Indomethacin caused severe lesions in the small intestine, mainly the jejunum and ileum, accompanied by intestinal hypermotility, the up-regulation of inducible nitric oxide synthase (iNOS) expression, and an increase of myeloperoxidase (MPO) activity as well as enterobacterial invasion in the mucosa. These events were all prevented by both $dmPGE_2$ and ampicillin, except the intestinal hypermotility which was only prevented by $dmPGE_2$. Likewise, irsogladine also significantly and dose-dependently prevented these lesions at >1 mg/kg. This agent alone increased mucus secretion and significantly suppressed the decreased mucus response to indomethacin, resulting in a suppression of the bacterial invasion as well as the increase in MPO activity and iNOS expression. The protective effect of irsogladine was mimicked by isobutylmethylxanthine, a nonselective inhibitor of phosphodiesterase (PDE), as well as rolipram, a selective PDE4 inhibitor. These results suggest that irsogladine protects the small intestine against indomethacin-induced lesions, and this effect may be associated with the increased mucus secretion, probably due to the inhibitory actions of PDE, resulting in suppression of enterobacterial invasion and iNOS expression.

Keywords Indomethacin-induced small intestinal lesion · Irsogladine · Mucosal protective drug · Phosphodiesterase type IV · Rat

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin are known to cause damage in the small intestine as well as the stomach, although the ulcerogenic dose is much lower in the former [1–3]. A number of factors such as intestinal hypermotility, bacterial flora, neutrophils, nitric oxide (NO), and inducible nitric oxide synthase (iNOS) are involved in the pathogenesis of these intestinal lesions [3–7], yet a deficiency of endogenous prostaglandins (PGs) due to cyclooxygenase (COX) inhibition is a most important base for the ulcerogenic response to NSAIDs. Recent studies including our own [8–10] showed that gastrointestinal ulcerogenic properties of NSAIDs are not solely explained by the inhibition of COX-1 and require the inhibition of both COX-1 and COX-2. 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. Unfortunately, antisecretory drugs such as proton pump inhibitors and histamine H_2 receptor antagonists are reported to be ineffective against NSAID-induced small intestinal lesions [11, 12], though these drugs are known to prevent gastric ulcerogenic responses to NSAIDs [13].

Irsogladine (2,4-diamino-6-[2,5-dichlorophenyl]-s-triazine maleate), a widely used anti-ulcer drug in Japan, is known to protect the gastric mucosa by enhancing the mucosal integrity of the stomach through facilitation of gap junctional intracellular communication [14, 15]. It has recently been shown that irsogladine increases the mucosal defensive ability in the stomach by increasing the intracellular levels of 3′,5′-cyclic adenosine monophosphate (cAMP) through inhibition of phosphodiesterase (PDE), especially type 4 [16]. Yet, it remains unknown whether irsogladine affords a prophylactic effect on indomethacin-induced small intestinal lesions.

In this study, we examined the effect of irsogladine on the ulcerogenic response induced by indomethacin in the rat small intestine, in comparison with other mucosal protective drugs such as rebamipide [17] and teprenone [18] as well as PDE inhibitors such as isobutylmethylxanthine (IBMX) and rolipram, and further investigated the mechanisms involved in the protective action of this agent.

Materials and Methods

Animals

Male Sprague–Dawley rats (200–260 g; Nippon Charles River, Shizuoka, Japan) were used. Studies were carried out using four to nine animals without fasting in a conscious state, unless otherwise specified. All experimental procedures described here were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

Induction of Small Intestinal Lesions

Animals were administered indomethacin (10 mg/kg, s.c.) and sacrificed 24 h later under deep ether anesthesia. Then, the small intestine was excised, treated with 2% formalin for fixation of tissue walls for 10 min, and opened along the antimesenteric attachment. The area of macroscopically visible damage (in units of mm^2) was measured under a dissecting microscope with square grids (10×), summed per tissue, and used as a lesion score. Irsogladine (1–10 mg/kg), rebamipide (100 and 300 mg/kg), teprenone (100 and 300 mg/kg), or $dmPGE_2$ (0.03 mg/kg) was given p.o. twice, 0.5 before and 6 h after the administration of indomethacin. Ampicillin (800 mg/kg) was given s.c. twice, 18 and 0.5 h before indomethacin. The nonselective PDE inhibitor IBMX (3 mg/kg) or selective PDE4 inhibitor rolipram (3 mg/kg) was given s.c. twice, 0.5 before and 6 h after the administration of indomethacin. The individual measuring the lesions was not aware of the treatments given to the animals.

Determination of Enterobacterial Counts

Enterobacteria were enumerated according to a method described by Deitch et al. [19]. Twenty-four hours after indomethacin treatment (10 mg/kg), the animals were sacrificed under deep diethyl 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 Gifu anaerobic medium agar (Nissui, Tokyo, Japan). Blood agar plates were incubated at 37°C for 24 h under aerobic conditions, whereas Gifu anaerobic medium agar plates were incubated for 24 h under standard anaerobic conditions (BBL Gas Pack Pouch Anaerobic System; BD Biosciences, San Jose, CA). Plates containing 10–300 colony-forming units (CFU) were examined for numbers of enterobacteria, and the data were expressed as log CFU per gram of tissue. Irsogladine (10 mg/kg), $dmPGE_2$ (0.03 mg/kg), rebamipide (100 mg/kg), or teprenone (300 mg/kg) was given p.o. twice, 30 min before and 6 h after the administration of indomethacin. Ampicillin (800 mg/kg) was given s.c. twice, 18 and 0.5 h before indomethacin.

Determination of MPO Activities

Myeloperoxidase (MPO) activity was measured according to a modified version of the method of Castro et al. [20]. The animals were sacrificed under deep ether anesthesia. All blood was withdrawn from the heart by perfusing with saline, and the large intestine was excised and opened along the antimesenteric attachment. After the tissue was rinsed with cold saline, the mucosa was scraped with glass slides, weighed, and homogenized in a 50-mmol phosphate buffer containing 0.5% hexadecyl-trimethyl-ammonium bromide (pH 6.0; Sigma, St. Louis, MO). The homogenized samples were subjected to freezing and thawing three times and centrifuged at 2,000 rpm for 10 min at 4°C. MPO activity in the supernatant was determined by adding 100 µl of the supernatant to 1.9 ml of 10 mmol phosphate buffer (pH 6.0) and 1 ml of 1.5 mol o-dianisidine hydrochloride (Sigma) containing 0.0005% w/v hydrogen peroxide. The changes in absorbance at 450 nm of each sample were recorded on a Hitachi spectrophotometer (U-2000, Hitachi, Ibaraki, Japan). Sample protein content was estimated by spectrophotometric assay (Protein Assay Kit, Pierce, IL, USA). The MPO activity was obtained from the slope of the reaction curve based on the following equation: specific activity (µmol H_2O_2/min/mg protein) = ((OD/min)/OD/µmol H_2O_2 × mg protein). Irsogladine (10 mg/kg), $dmPGE_2$ (0.03 mg/kg), rebamipide (100 mg/kg), or teprenone

(300 mg/kg) was given p.o. twice, 30 min before and 6 h after the administration of indomethacin. Ampicillin (800 mg/kg) was given s.c. twice, 18 and 0.5 h before indomethacin.

Determination of iNOS mRNA Expression by RT-PCR

Expression of iNOS mRNA in the small intestinal mucosa was measured by RT-PCR. The animals were sacrificed under deep ether anesthesia 6 h after the administration of indomethacin (10 mg/kg), and the small intestines were removed, frozen in acetone/dry ice, and stored at −80°C prior to use. The tissue samples were pooled from two or three rats for extraction of total RNA, which was achieved by a single-step acid phenol-chloroform extraction procedure using TRIZOLE (GIBCO BRL, Gaithersburg, MD). Total RNA primed by random hexadeoxy ribonucleotide was reverse-transcribed with the SUPERSCRIPT preamplification system (GIBCO BRL). The sequences of sense and antisense primers for the rat iNOS were described in Table 1. An aliquot of the RT reaction product served as a template in 32 cycles with 0.5 min of denaturation at 95°C and 1 min of extension at 68°C using the Advantage 2 polymerase mixture (CLONTECH) on a thermal cycler (TAKARA TP-240). A portion of the PCR mixture was electrophoresed in 1.8% agarose gel in TAE buffer (40 mM Tris, 2 mM EDTA, and 20 mM acetic acid; pH: 8.1), and the gel was stained with ethidium bromide and photographed. Irsogladine (10 mg/kg), $dmPGE_2$ (0.03 mg/kg), rebamipide (100 mg/kg), or teprenone (300 mg/kg) was given p.o. 30 min before indomethacin treatment, while ampicillin (800 mg/kg) was given s.c. twice, 18 and 0.5 h before indomethacin. The effect of irsogladine (10 mg/kg) alone on iNOS expression was also examined in the small intestine of normal rats without indomethacin treatment.

Measurement of Small Intestinal Motility

Intestinal motility was measured according to a modified version of the method of Calignano et al. [21]. In brief, the rat was 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 mm Hg. After the preparation was allowed to rest for 30 min, 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, Ibaragi, Japan). Indomethacin (10 mg/kg) was given s.c. after basal intestinal motility had well stabilized, and the motility was measured for 3 h thereafter. Irsogladine (10 mg/kg), rebamipide (100 mg/kg), teprenone (300 mg/kg), $dmPGE_2$ (0.03 mg/kg), or ampicillin (800 mg/kg) was given intraduodenally (i.d.) 2 h after the administration of indomethacin.

Table 1 PCR primer sequences and product size

GAPDH	
Sense: 5′-GAACGGGAAGCTCACTGGCATGGC-3′	310 bp
Antisense: 5′-TGAGGTCCACCACCCTGTTGCTG-3′	
iNOS	
Sense: 5′-CGGTTCACAGTCTTGGTGAAAG-3′	651 bp
Antisense: 5′-CAGGTGTTCCCCAGGTAGGTAG-3′	

Determination of Mucus Secretion in Small Intestine

The amount of mucus secreted in the small intestine was determined by periodic acid-Schiff (PAS) staining. Three hours after the administration of indomethacin (10 mg/kg, s.c.), the animals were sacrificed 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 h, embedded in paraffin, and sectioned at a thickness of 8 μm. PAS staining was subsequently performed according to the conventional method. Irsogladine (10 mg/kg) was given p.o. with or without co-administration of indomethacin. In the combined administration experiment, this agent was given p.o. 30 min before the administration of indomethacin.

Preparation of Drugs

The drugs used were irsogladine maleate (Nippon Shinyaku Co., Ltd., Kyoto, Japan), indomethacin, ampicillin, 3-isobutyl-1-methylxanthine (IBMX), rolipram (Sigma), 16,16-dimethyl PGE_2 (Funakoshi), teprenone (Eisai, Tokyo, Japan; 6,10,14,18-tetramethyl-5,9,13,17-nonadectatetraen 2-one), and rebamipide (Ohtsuka, Tokushima, Japan; 2-(4-chlorobenzoylamino)-3-[2(1H)–quinolinon-4-yl] propionic acid). Indomethacin, IBMX, or rolipram was suspended in saline with a drop of Tween 80 (Wako, Osaka, Japan). Irsogladine was suspended in a 0.5% hydroxy propyl cellulose solution (Wako). Ampicillin was dissolved in saline. All drugs were prepared immediately before use and administered p.o., s.c., or i.p. in a volume of 0.5 ml/100 g body weight.

Statistics

Data are presented as the mean ± SE for four to nine 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

Effect of Irsogladine on Indomethacin-Induced Small Intestinal Lesions

Subcutaneously administered indomethacin (10 mg/kg) in normally fed rats produced multiple hemorrhagic lesions in the small intestine, mainly in the jejunum and ileum, with an ulcer score of 223.3 ± 35.3 mm^2. Irsogladine, given orally at 1–10 mg/kg, dose-dependently prevented the development of these lesions in response to indomethacin, and the effect was significant at 1 mg/kg or greater; the lesion score at 3 mg/kg was 38.9 ± 17.4 mm^2, and the degree of inhibition was 82.6% (Fig. 1). Likewise, both $dmPGE_2$ (0.03 mg/kg) and ampicillin (800 mg/kg) significantly reduced the severity of small intestinal lesions, with lesion scores of 52.0 ± 17.0 and 23.7 ± 7.8 mm^2, respectively. On the other hand, the development of indomethacin-induced intestinal lesions was also significantly prevented by oral administration of rebamipide (100 mg/kg) and teprenone (300 mg/kg), with degrees of inhibition of 70.2% and 56.2%, respectively.

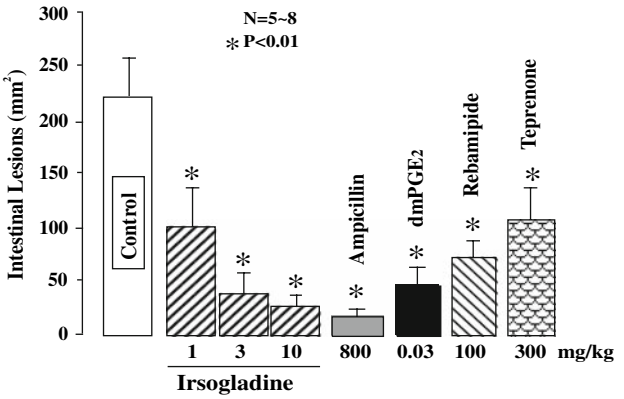

Fig. 1 Effects of irsogladine, rebamipide, teprenone, $dmPGE_2$ and ampicillin on indomethacin-induced small intestinal lesions in rats. The animals were given indomethacin (10 mg/kg, s.c.) and sacrificed 24 h later. Irsogladine (1–10 mg/kg), rebamipide (100 mg/kg), teprenone (300 mg/kg), or $dmPGE_2$ (0.03 mg/kg) was given p.o. twice, 30 min before and 6 h after the administration of indomethacin, while ampicillin (800 mg/kg) was given s.c. twice, 18 and 0.5 h before indomethacin. Data are presented as the mean ± SE for 5–8 rats. *Significant difference from control at $P < 0.01$

Effect of Irsogladine on MPO Activity in Small Intestinal Mucosa

The MPO activity in the normal intestinal mucosa was 0.009 ± 0.006 μmol H_2O_2/mg protein and markedly elevated in response to indomethacin (10 mg/kg), reaching 0.088 ± 0.025 μmol H_2O_2/mg protein 24 h later (Fig. 2). The elevated MPO activity was almost totally suppressed by irsogladine at 10 mg/kg, and the value was 0.009 ± 0.005 μmol H_2O_2/mg protein, with an inhibition of 89.8%. Likewise, both $dmPGE_2$ (0.03 mg/kg) and ampicillin (800 mg/kg) also significantly prevented the increase of MPO activity following indomethacin treatment, with inhibitions of 84.4% and 97.4%, respectively. Both rebamipide (100 mg/kg) and teprenone (300 mg/kg) also significantly suppressed the rise in MPO activity caused by indomethacin treatment, yet the effect was less potent than that of irsogladine, with inhibitions of 65.0% and 51.4%, respectively.

Effect of Irsogladine on Mucosal Invasion of Enterobacteria Caused by Indomethacin

The aerobic and anaerobic bacterial counts in the normal intestinal mucosa were 5.94 ± 0.16 log CFU/g tissue and 6.78 ± 0.22 log CFU/g tissue, respectively. Following subcutaneous administration of indomethacin (10 mg/kg), the bacterial counts in both aerobic and anaerobic

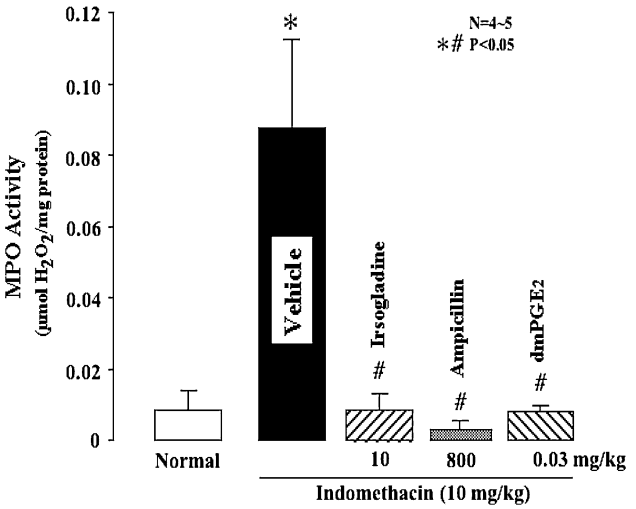

Fig. 2 Effects of irsogladine and ampicillin on the MPO activity in rat small intestine. The animals were given indomethacin (10 mg/kg, s.c.) and sacrificed 24 h later. Irsogladine (10 mg/kg, p.o.) or $dmPGE_2$ (0.03 mg/kg) was given twice, 30 min before and 6 h after the administration of indomethacin, while ampicillin (800 mg/kg) was given p.o. twice, 18 and 30 min before indomethacin. Data are presented as the mean ± SE for 4–5 rats. Significant difference at $P < 0.05$ (* from normal, # from vehicle)

conditions were markedly increased and reached values of 8.74 ± 0.18 log CFU/g tissue and 8.80 ± 0.23 log CFU/g tissue, respectively, 24 h later (Table 2). Pretreatment of the animals with irsogladine (10 mg/kg) significantly prevented bacterial invasion in the mucosa following the administration of indomethacin, although this agent alone had no effect on bacterial counts in the normal intestinal mucosa. As expected, both ampicillin (800 mg/kg) and $dmPGE_2$ (0.03 mg/kg) significantly prevented the mucosal invasion of enterobacteria in response to indomethacin, especially in the animals treated with the former in which the bacterial count decreased to even lower than that of control rats without indomethacin treatment. Likewise, the indomethacin-induced bacterial invasion was also significantly prevented by rebamipide (100 mg/kg) or teprenone (300 mg/kg), though the effect was much less potent than that of irsogladine (not shown).

Effect of Irsogladine on Mucosal Expression of iNOS mRNA

Expression of iNOS mRNA was barely detected in the normal intestinal mucosa, but markedly up-regulated after the administration of indomethacin (10 mg/kg) when examined 6 h later (Fig. 3). The up-regulation of iNOS expression caused by indomethacin was almost totally inhibited by pretreatment of the animals with irsogladine (10 mg/kg), though this agent had no effect in the normal rat intestine. As expected, both $dmPGE_2$ (0.03 mg/kg) and ampicillin (800 mg/kg) prevented the expression of iNOS mRNA in the intestinal mucosa following indomethacin treatment (Fig. 4a). Likewise, an apparent suppression of iNOS expression was also observed on the prior administration of rebamipide (100 mg/kg) or teprenone (300 mg/kg) (Fig. 4b).

Effect of Irsogladine on Intestinal Hypermotility Response to Indomethacin

Subcutaneous administration of indomethacin (10 mg/kg) caused a marked increase in small intestinal motility from about 30 min after the administration (Fig. 5). Irsogladine (10 mg/kg) given i.d. did not affect the intestinal hypermotility induced by indomethacin. Consistent with our previous findings [4], the intestinal hypermotility in response to indomethacin was markedly inhibited by i.d. administration of $dmPGE_2$ (0.03 mg/kg) but not ampicillin (800 mg/kg). In addition, neither rebamipide (100 mg/kg) nor teprenone (300 mg/kg) had any effect on indomethacin-induced intestinal hypermotility (not shown).

Effect of Irsogladine on PAS-Positive Substances in Small Intestine

In the normal intestinal mucosa, PAS-positive substances were clearly observed over the surface epithelial cells and along the glands (Fig. 6a). Indomethacin (10 mg/kg) apparently reduced the amount of PAS-positive substances on the epithelial cells as well as in the glands (Fig. 6c). However, the reduction in PAS staining was attenuated when irsogladine (10 mg/kg) was administered with indomethacin (Fig. 6d). Administration of irsogladine alone also increased the amount of PAS-positive substances in the mucosa when compared to the control mucosa (Fig. 6b).

Effects of PDE Inhibitors on Small Intestinal Lesions Induced by Indomethacin

Multiple hemorrhagic lesions developed in the small intestine within 24 h after the administration of indomethacin

Table 2 Effect of irsogladine on bacterial counts in rat small intestine with or without indomethacin treatment

Group	Number of rats	Number of bacteria (log CFU/g tissue)	
		Aerobic	Anaerobic
Normal	6	5.94 ± 0.16	6.78 ± 0.22
Irsogladine	6	6.10 ± 0.02	7.27 ± 0.17
Indomethacin			
+Vehicle	7	8.74 ± 0.18*	8.80 ± 0.23*
+Irsogladine	6	6.88 ± 0.43**	7.30 ± 0.14**
+Ampicillin	5	4.97 ± 0.38**	4.82 ± 0.32**
+16,16-$dmPGE_2$	5	6.36 ± 0.38**	7.06 ± 0.32**

The animals were given indomethacin (10 mg/kg, s.c.) and sacrificed 24 h later. Irsogladine (10 mg/kg, p.o.) was given twice, 30 min before and 6 h after the administration of indomethacin, while ampicillin (800 mg/kg) was given p.o. twice, 24 h and 30 min before indomethacin. Data are presented as the mean ± SE for 6–7 rats. Significant difference at $P < 0.01$ (* from normal, ** from indomethacin +vehicle)

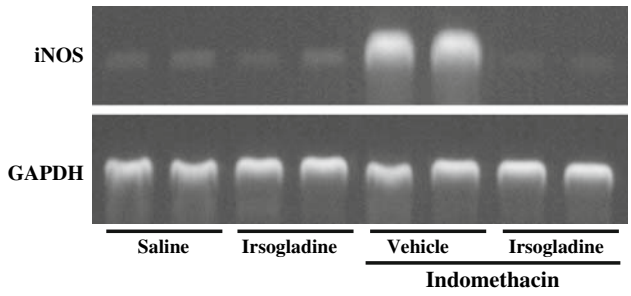

Fig. 3 Effect of irsogladine on the expression of iNOS mRNA in rat small intestine following indomethacin treatment. The animals were given indomethacin (10 mg/kg, s.c.) and sacrificed 6 h later, and the expression of iNOS was examined by RT-PCR. Irsogladine (10 mg/kg) was given orally 30 min before the administration of indomethacin. The effect of irsogladine on iNOS expression in the normal rat intestine without indomethacin treatment was also examined

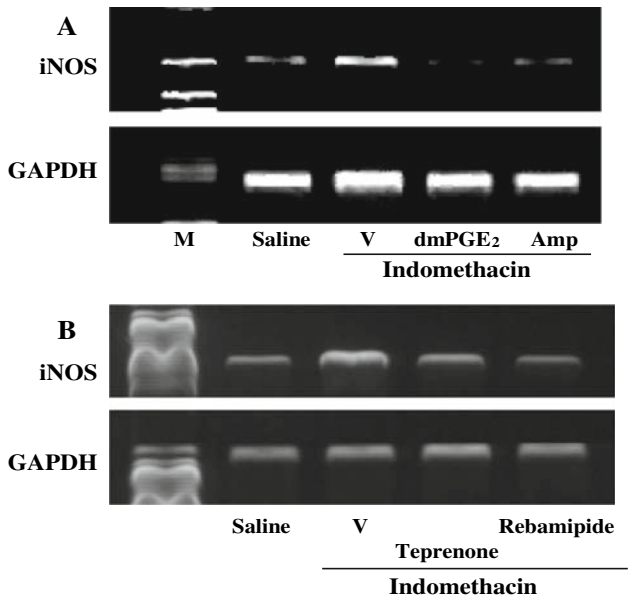

Fig. 4 Effects of ampicillin (**A**), $dmPGE_2$ (**A**), rebamipide (**B**), and teprenone (**B**) on the expression of iNOS mRNA in rat small intestine following indomethacin treatment. The animals were given indomethacin (10 mg/kg, s.c.) and sacrificed 6 h later, and the expression of iNOS was examined by RT-PCR. $DMPGE_2$ (0.03 mg/kg), rebamipide (10 mg/kg), or teprenone (300 mg/kg) was given orally 30 min before the administration of indomethacin, while ampicillin (800 mg/kg) was given p.o. twice, 18 and 30 min before indomethacin. *V* vehicle

(10 mg/kg). Pretreatment of the animals with IBMX (3 mg/kg), a nonselective PDE inhibitor, almost totally prevented the development of these lesions in response to indomethacin, with a lesion score of 15.8 ± 6.8 mm^2 (Fig. 7). Likewise, the PDE4 selective inhibitor rolipram (3 mg/kg) also potently inhibited these lesions, similar to IBMX, with a lesion score of 22.5 ± 5.6 mm^2.

Discussion

Irsogladine, a widely used anti-ulcer drug in Japan, is known to increase the resistance of the gastric mucosa to noxious stimuli. This study showed that irsogladine protected the small intestinal mucosa against indomethacin-induced lesions, together with the suppression of inflammatory responses. Furthermore, this agent prevented enterobacterial invasion as well as the expression of iNOS mRNA in the mucosa following indomethacin treatment, the major pathogenic events in the ulcerogenic response.

NSAIDs such as indomethacin cause damage in the small intestine as well as the stomach [1–3]. Recent studies showed an up-regulation of COX-2 expression in these tissues after the administration of NSAIDs and demonstrated that these ulcerogenic properties of NSAIDs require the inhibition of both COX-1 and COX-2 [8–10]. It is also known that NSAIDs cannot induce damage in the small intestine of germ-free animals or even fasting rats [22, 23], suggesting that the presence of enterobacteria is essential for the development of intestinal lesions. Because enterobacteria release endotoxin (lipopolysaccharide [LPS]), which causes the up-regulation of iNOS expression and overproduction of NO in the gut mucosa [23, 24], it is believed that NO plays a pathogenic role in the intestinal ulcerogenic response induced by NSAIDs. Indeed, indomethacin-induced intestinal lesions were prevented by aminoguanidine, a selective iNOS inhibitor, as well as dexamethasone, an inhibitor of iNOS induction [23]. Considering all these, it is assumed that the up-regulation of iNOS expression is causally related to COX inhibition and plays a pathogenic role in NSAID-induced intestinal lesions.

First, this study clearly showed that irsogladine prevented the development of intestinal lesions in response to indomethacin. The effect was dependent on the dose and was significant even at 1 mg/kg. We confirmed that both rebamipide and teprenone at much higher doses afforded protection against these lesions, yet were much less effective than irsogladine. Certainly, indomethacin-induced intestinal damage was significantly prevented by pretreatment of the animals with $dmPGE_2$ as a supplement for PG deficiency or ampicillin as an antibiotic.

NSAID-induced intestinal lesions do not occur in germ-free animals or fasting animals [22], suggesting a major pathogenic role for enterobacteria in this model. This idea was supported by the finding that indomethacin-induced intestinal lesions were prevented by prior administration of ampicillin. As shown in our previous and present studies, indomethacin caused bacterial invasion in the mucosa, followed by iNOS expression and NO production as well as neutrophil recruitment, and these processes were all hampered by this antibiotic [23]. Because irsogladine

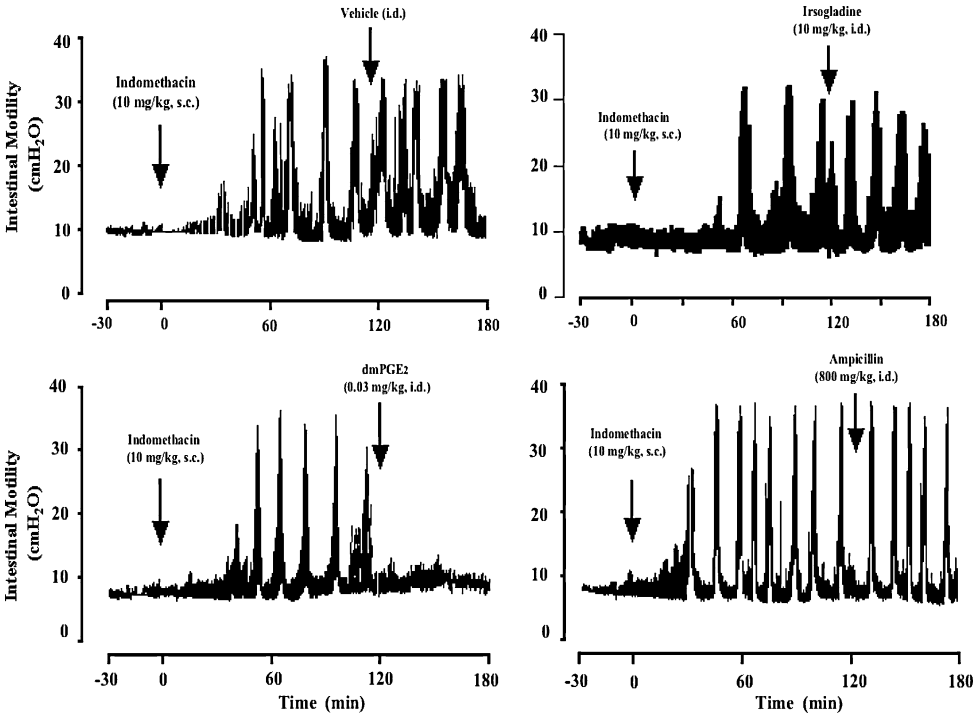

Fig. 5 Effects of irsogladine, $dmPGE_2$, and ampicillin on small intestinal hypermotility caused by indomethacin in a rat. Indomethacin (10 mg/kg) was given s.c., and small intestinal motility was recorded for 3 min thereafter. Irsogladine (10 mg/kg), $dmPGE_2$ (0.03 mg/kg), or ampicillin (800 mg/kg) was given i.d. 2 h after the administration of indomethacin

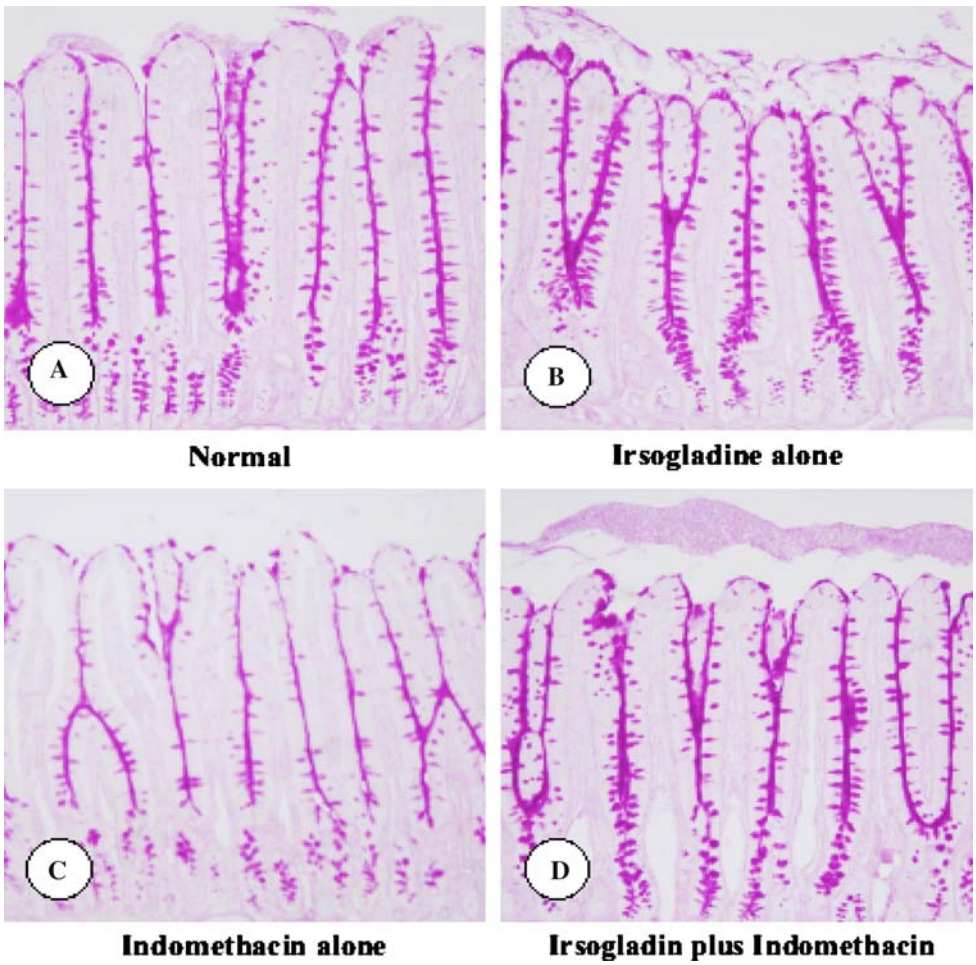

Fig. 6 Microscopic observation of the rat small intestinal mucosa. The animals were given indomethacin (10 mg/kg) s.c. and sacrificed 3 h later. Irsogladine (10 mg/kg) was given orally with or without the co-administration of indomethacin. In the combined administration, irsogladine was given 30 min before the administration of indomethacin. Figure shows normal (**A**), irsogladine alone (**B**), indomethacin alone (**C**), irsogladine plus indomethacin (**D**) (PAS; 200×)

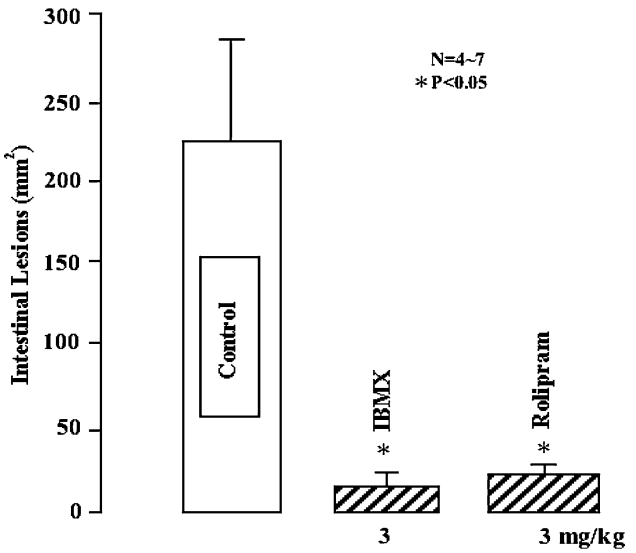

Fig. 7 Effects of IBMX and rolipram on indomethacin-induced small intestinal lesions in rats. The animals were given indomethacin (10 mg/kg, s.c.) and sacrificed 24 h later. IBMX (3 mg/kg) or rolipram (3 mg/kg) was given i.p. twice, 30 min before and 6 h after the administration of indomethacin. Data are presented as the mean ± SE for 4–7 rats. * Significant difference from control at $P < 0.01$

significantly mitigated the bacterial invasion in the mucosa following indomethacin treatment, this effect may account, at least partly, for the prophylactic effect of this agent against indomethacin-induced intestinal lesions. As expected, enterobacterial invasion under indomethacin treatment was also significantly prevented by both rebamipide and teprenone, consistent with our previous observations [25, 26].

This study confirmed that indomethacin markedly enhanced intestinal motility and this functional response occurred much earlier than the development of intestinal damage [4]. Abnormal hypermotility caused by indomethacin results in a disruption of the unstirred mucus layer, through vigorous mixing with food residue in the lumen, leading to facilitation of bacterial invasion in the mucosa [24]. As shown in this study, the intestinal hypermotility was inhibited by $dmPGE_2$ but not ampicillin. This prostanoid, through inhibition of intestinal hypermoitlity via the activation of EP4 receptors [27], may strengthen the mucus barrier against luminal pathogens, resulting in suppression of bacterial invasion following indomethacin treatment. Certainly, ampicillin also decreased the bacterial count in the mucosa because of its sterilizing action and prevented the intestinal ulcerogenic response, despite having no effect on the hypermotility in response to indomethacin. Irsogladine did not affect the intestinal hypermotility caused by indomethacin, yet it significantly prevented bacterial invasion and intestinal ulceration following indomethacin treatment.

The mechanism by which irsoglandine prevented bacterial invasion following indomethacin treatment remains unknown. As mentioned, mucus plays an important role in the innate host defence against intestinal pathogens and irritants. We showed in the present study that irsogladine by itself markedly increased levels of PAS-positive materials in the intestinal mucosa and hampered the reduced response to indomethacin. It is known that PGE_2 also promotes the secretion of mucus through the activation of EP4 receptors in the gastrointestinal tract including the small intestine [28]. EP4 receptors, coupled with Gs proteins, cause an increase in the intracellular level of cAMP through stimulation of adenylate cyclase [29]. A recent study showed that irsogladine protected the gastric mucosa by increasing intracellular levels of cAMP through inhibition of PDE activity, especially PDE4 activity [16]. We found that the intestinal ulcerogenic response to indomethacin was markedly prevented by the nonselective PDE inhibitor IBMX as well as the selective PDE4 inhibitor rolipram. Irsogladine also reportedly suppressed neutrophil activation through an increase of intracellular cAMP due to inhibition of PDE4 [16]. It is possible that irsogladine promotes the secretion of mucus mediated intracellularly by cAMP through the inhibition of PDE4 activity. Likewise, both rebamipide and teprenone are also known to stimulate the secretion of mucus in the gastrointestinal tract [30, 31]. Thus, the inhibitory effects of these agents on bacterial invasion following indomethacin treatment may somehow be attributable to an increase in the production/secretion of mucus in the small intestine.

It should be noted in this study that irsogladine prevented the up-regulation of iNOS expression in the small intestine, an important event in the occurrence of intestinal lesions caused by NSAIDs [23]. We previously showed that iNOS expression in the small intestine results from COX-1 inhibition and is functionally related to intestinal hypermotility and bacterial invasion [23, 32]. This idea was supported by the findings that this event was similarly induced by SC-560, the selective COX-1 inhibitor, and inhibited by pretreatment with $dmPGE_2$ and ampicillin as well as atropine [32]. Since irsogladine was found to prevent bacterial invasion, probably by stimulating the secretion of mucus, it would be understandable that this agent suppressed the expression of iNOS in the small intestine following indomethacin treatment. As expected, both rebamipide and teprenone also prevented the up-regulation of iNOS expression after the administration of indomethacin. These results taken together support a cause–effect relationship between stimulation of mucus secretion, prevention of bacterial invasion, and suppression of iNOS expression. Certainly, there are other mechanisms involved in the inhibitory effect of irsogladine on iNOS expression after indomethacin treatment. Bertrard et al.

[33] reported that indomethacin increased the release of tumor necrosis factor-α (TNF-α) in the small intestinal mucosa, especially in the area where bacterial invasion was encountered. Since TNF-α mediates the up-regulation of iNOS expression caused by endotoxin and since the release of TNF-α is suppressed by cAMP [34, 35], it is possible that the inhibitory effect of irsogladine on iNOS expression is brought about, at least partly, by an increase of cAMP due to inhibition of PDE4.

Irsogladine did not have any effect on the intestinal hypermotility in response to indomethacin. Since cAMP is known to relax smooth muscle in the gastrointestinal tract, it is expected that irsogladine also prevents intestinal hypermotility by increasing the intracellular cAMP level through inhibition of PDE4. At present, the reason why irsogladine did not affect intestinal hypermotility remains unknown. Mere speculation indicates that different subtypes of PDE may be associated with intestinal functions of different cells, such as the secretion of mucus by the surface cells and motility of smooth muscle. If PDE4 is mainly expressed in mucus cells and not smooth muscle cells, then it is possible that irsogladine affects only the production of mucus, not smooth muscle contraction. This point should certainly be verified in a future study.

Taking all the present and previous findings together, it is concluded that irsogladine protects the small intestine against indomethacin-induced lesions, and this prophylactic effect may be associated with the increased secretion of mucus, probably due to its inhibitory action of PDE4, resulting in suppression of enterobacterial invasion and iNOS expression.

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