PROTECTION BY DRUGS OF EXPERIMENTAL INTESTINAL LESIONS

Protective effect of rebamipide on indomethacin-induced intestinal damage in rats

HIROYUKI MIZOGUCHI, YOSHIHIRO OGAWA, KENJI KANATSU, AKIKO TANAKA, SHINICHI KATO AND KOJI TAKEUCHI

Department of Pharmacology and Experimental Therapeutics, Kyoto Pharmaceutical University, Yamashina, Kyoto, Japan

Abstract

Background and Aim: We evaluated the effect of rebamipide (2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinon-4-yl] propionic acid), a novel anti-ulcer drug, on indomethacin-induced small intestinal lesions in rats.

Methods: The animals were administered indomethacin (10 mg/kg, s.c.), and they were killed 24 h later. Rebamipide (30–300 mg/kg) was administered p.o. twice, 30 min before, and 6 h after indomethacin.

Results: Indomethacin caused hemorrhagic lesions in the rat small intestine, accompanied by an increase in enterobacterial translocation, inducible nitric oxide synthase (iNOS) and myeloperoxidase (MPO) activities, as well as thiobarbituric acid (TBA) reactants, and these changes were significantly prevented by the supplementation with 16,16-dimethyl prostaglandin E_2 (dmPGE2; 10 µg/kg, i.v.) or the pretreatment of animals with the antibiotic ampicillin. Treatment of the animals with rebamipide dose-dependently prevented the development of intestinal lesions, and this effect was mimicked by i.v. administration of superoxide dismutase (SOD: 3000 U/kg) + catalase (CAT: 5000 U/kg). The protection by rebamipide was accompanied by a significant suppression of the increase in both MPO and iNOS activities, and a complete inhibition of the increase in TBA reactants, while SOD + CAT significantly inhibited the increase of MPO activity and TBA reactants, but not iNOS activity. The bacterial translocation following indomethacin was also significantly decreased by either rebamipide or SOD + CAT.

Conclusion: These results confirmed the importance of enterobacteria and iNOS/NO in the pathogenesis of indomethacin-induced small intestinal lesions, and suggested that rebamipide prevents the development of these lesions, probably by its radical scavenging action.

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Key words: indomethacin, neutrophil radical scavenging action, rat, rebamipide, small intestinal damage.

INTRODUCTION

Administration of non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin to humans or experimental animals causes ulceration in the small intestine as well as the stomach as adverse effects.[1–3] Several factors have been postulated as the pathogenic element of indomethacin-induced intestinal ulceration, including bacterial translocation, neutrophil activation and nitric oxide (NO), in addition to prostaglandin (PG) deficiency.[4–10] Enterobacteria and NO especially play a key pathogenic role in this ulcer model; the release of bacterial products such as endotoxin contributes to the development of intestinal damage through the overproduction of NO by the upregulation of inducible NO synthase (iNOS) in the mucosa.[9,11] Furthermore, it is considered that a detrimental role of NO in this lesion model is explained by a cytotoxic effect of peroxynitrite produced from NO in the presence of superoxide radicals.[9,10,12]

Rebamipide (2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinon-4-yl] propionic acid), a novel anti-ulcer drug, has been reported to prevent various acute experimental gastric lesions, and accelerate the healing of chronic gastric ulcers.[13,14] Recent studies have also shown the beneficial effect of this drug on experimentally induced colitis.[15,16] Several reports have been published concerning the mechanism by which rebamipide results in the mucosal protection, including stimulation of mucus secretion,[17] PG biosynthesis,[18] a radical scavenging action,[19–21] and a heat shock protein.[22] However, there is no study examining the effect of rebamipide on indomethacin-induced small intestinal lesions.

Given the above background, we evaluated in the present study, the prophylactic effect of rebamipide on indomethacin-induced small intestinal lesions in rats, in comparison with that of superoxide dismutase (SOD) plus catalase (CAT). Because 16,16-dimethyl prostaglandin E_2 (dmPGE$_2$) or ampicillin prevents indomethacin-induced intestinal lesions by supplementation of PG deficiency or inhibition of bacterial translocation, respectively,[12] we used these drugs as the standard prophylactic treatment to search for the possible mechanism involved in the protective action of rebamipide.

METHODS

Animals

Male Sprague–Dawley rats (200–230 g; Nippon Charles River, Shizuoka, Japan) were used. The experiments were performed by using five to six non-fasting rats per group under unanesthetized conditions, unless otherwise specified. All experimental procedures used in the present study were approved by the Experimental Animal Research Committee of the Kyoto Pharmaceutical University.

General procedures

The animals were administered indomethacin s.c. in a dose of 10 mg/kg, and the small intestinal mucosa was examined 24 h later for damage, bacterial translocation, and other biochemical parameters such as the enterobacterial number, myeloperoxidase (MPO) activity, iNOS activity and lipid peroxidation represented by the production of thiobarbituric acid (TBA) reactants. By using this lesion model, the effects of rebamipide and SOD + CAT on the intestinal ulcerogenic responses to indomethacin were examined. We also examined the effects of (dmPGE$_2$) and ampicillin as reference drugs on this lesion model. Rebamipide (30–300 mg/kg) was given p.o. twice, 30 min before and 6 h after administration of indomethacin, while SOD (3000 U/kg) + CAT (5000 U/kg) or dmPGE$_2$ (10 μg/kg) was given i.v. twice 30 min before and 6 h after indomethacin. Ampicillin (800 mg/kg) was given p.o. twice, 24 h and 30 min before indomethacin treatment. The doses of the drugs used were selected according to the previously published papers.[10,12,16,19,23]

Induction of small intestinal ulceration

The animals were administered indomethacin (10 mg/kg) s.c., killed 24 h later under deep ether anesthesia, and both the jejunum and ileum were removed and treated with 2% formalin for fixation of the tissues.[10,12] They were then opened along the mesenteric attachment and examined for lesions under a dissecting microscope with square grids (×10). The area (mm^2) of macroscopically visible lesions was measured, summed per small intestine, and used as a lesion score. The person measuring the lesions did not know the treatments given to the animals.

Measurement of bacterial translocation

Enterobacterial translocation was measured according to the previously published methods.[11,23] The animals were killed under deep ether anesthesia, and the small intestines were removed. After rinsing the intestine 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 wet tissue. The aliquot of the homogenate was placed on blood agar and Gifu University Anaerobic Medium (GAM) agar (Nissui, Osaka, Japan). Blood agar plates were incubated at 37°C for 24 h under aerobic conditions, while GAM agar plates were incubated for 48 h under standard anaerobic conditions (BBL GasPack Pouch Anaerobic System; Becton Deckinson, Cockeysville, MD, USA). Plates containing 10–200 colony-forming units (CFU) were examined for enterobacterial numbers that had invaded the small intestine, and the data were expressed as log CFU/g tissue.

Determination of myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured according to the modified method of Bradley *et al.*[24] In brief, the animals were killed under deep ether anesthesia, and the small intestines were removed. After rinsing the intestine with cold saline, the mucosa was scraped by using two glass slides, weighed, and homogenized in 50 mmol/L phosphate buffer containing 0.5% hexadecyl-trimethyl-ammonium bromide (HTAB; pH 6.0; Sigma Chemicals, St Louis, MO, USA). The homogenized samples were frozen and thawed three times, and centrifuged at 358 *g* for 10 min at 4°C. Myeloperoxidase activity in the supernatant was determined by adding 100 μL of the supernatant to 1.9 mL of 10 mmol/L phosphate buffer (pH 6.0) and 1 mL of 1.5 mol/L *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).

The sample protein content was estimated by using the spectrophotometric assay (Pierce protein assay kit; PIERCE, Rockford, IL, USA), and the MPO activity was obtained from the slope of the reaction curve, based on the following equation: Specific activity (μmol H_2O_2/min per mg protein) = ((OD/min)/OD/μmol $H_2O_2 \times$ mg protein).

Measurement of inducible nitric oxide synthase activity

Intestinal mucosal NOS activity was measured by determining the conversion of radiolabeled L-arginine to citrulline, according to the method described by Brown *et al.*[5] The animals were killed by deep ether anesthesia, and the small intestines were removed. After rinsing the intestine with cold saline, the mucosa was scraped, and homogenized in ice-cold buffer (Tris-HCl 50 mmol/L, sucrose 32 mmol/L, dithiothreitol 1 mmol/L, leupeptin 10 μg/mL and aprotinin 2 μg/mL), adjusted to pH 7.4 with NaOH, followed by centrifugation for 20 min at 8944 *g* at 4°C. The supernatant was incubated for 60 min at 37°C in reaction buffer comprising: Tris-HCl buffer 50 mmol/L, $CaCl_2$ 1.25 mmol/L, valine 12.5 mmol/L, dithiothreitol 1 mmol/L, L-arginine 10 μmol/L, NADPH 100 μmol/L, flavin adenine dinucleotide (FAD) 10 μmol/L, flavin mononucleotide (FMN) 10 μmol/L and [^{3}H]-L-arginine 18 500 Bq/mL. The reaction was arrested by removal of the substrate L-arginine with addition of aliquot of 50% suspension of Dowex, Tokyo, Japan (AG 50 W-8, Na + form) in water, and each sample was centrifuged at 805 *g* for 5 min After allowing the resin to settle, the supernatant was removed for estimation of the radiolabeled products by scintillation counting. The activity of constitutive NOS (cNOS) was determined from the difference between the presence and absence of 1 mmol/L *O,O-bis*(2-aminoethyl)ethyleneglycol-*N,N,N′-N″*-tetraacetic acid (EGTA); the activity of iNOS was determined in the presence of 1 mmol/L EGTA. Sample protein content was estimated by the spectrophotometric assay as described above, and the NOS activity was expressed as pmol/min per mg protein.

Determination of lipid peroxidation

The lipid peroxidation in the intestinal mucosa was determined as thiobarbituric acid (TBA) reactants, according to the modified method of Ohkawa *et al.*[25] Briefly, the animals were killed under deep ether anesthesia and the small intestine was removed. After rinsing the tissue with cold saline, the mucosa was scraped, weighed, and homogenized in 10 mL KCl. The homogenate was supplemented with the mixture of TBA and boiled at 100°C for 1 h. The reactants were then supplemented with 5 mL of the mixture of *n*-butanol and pyridine, shaken vigorously for 1 min and centrifuged for 10 min at 2800 *g*. Absorbance was measured at 532 nm on a Hitachi spectrophotometer (Hitachi, Ibaraki, Mito, Japan) and the results were expressed as nmol/TBA per mg protein.

Preparation of drugs

Drugs used were indomethacin, rebamipide (Ohtsuka Pharm. Co. Ltd, Tokushima, Japan), ampicillin (Sigma Chemicals), superoxide dismutase (SOD), catalase (CAT; Nacalai tesque, Kyoto, Japan) and 16,16-dimethyl prostaglandin E_2 (dmPGE$_2$: Funakoshi, Tokyo, Japan). Indomethacin was suspended in saline with a drop of Tween 80 (Wako, Osaka, Japan), while rebamipide was suspended in carboxymethylcellulose (CMC: Nacali Tesque) solution. 16,16-Dimethyl prostaglandin E_2 was first dissolved in absolute ethanol and then diluted with saline to a desired concentration. Other drugs were dissolved in saline. All drugs were prepared immediately before use and administered s.c. and p.o. in a volume of 0.5 mL/100 g bodyweight or i.v. in a volume of 0.1 mL/100 g bodyweight.

Statistics

Data are presented as the mean ± SE from five to six rats per group. Statistical analyses were performed by using a two-tailed Student's *t*-test or Dunnett's multiple comparison test, and values of $P < 0.05$ were regarded as significant.

RESULTS

Effect of rebamipide on small intestinal damage induced by indomethacin

A single administration of indomethacin subcutaneously at 10 mg/kg provoked severe hemorrhagic lesions in the small intestine, mostly the jejunum and ileum, the lesion score being 215.0 ± 22.2 mm^2 (Fig. 1). The development of intestinal lesions in response to indomethacin was dose-dependently prevented by rebamipide (30–300 mg/kg) given twice 30 min before and 6 h after indomethacin, the degree of inhibition at 100 mg/kg being 64.0%. Likewise, the development of small intestinal lesions in response to indomethacin was also significantly inhibited by the combined treatment with SOD (3000 U/kg) + CAT (5000 U/kg), the degree of inhibition being 57.0%. Certainly, when the animals were pretreated with dmPGE$_2$ (10 μg/kg) or ampicillin (800 mg/kg), these agents markedly reduced the severity of indomethacin-induced intestinal lesions, the degree of inhibition being 73.2 or 93.5%, respectively.

Effects of rebamipide on various changes in the small intestinal mucosa by indomethacin

The MPO activity was 0.04 ± 0.01 μmol H_2O_2/mg protein in the normal intestinal mucosa, and markedly
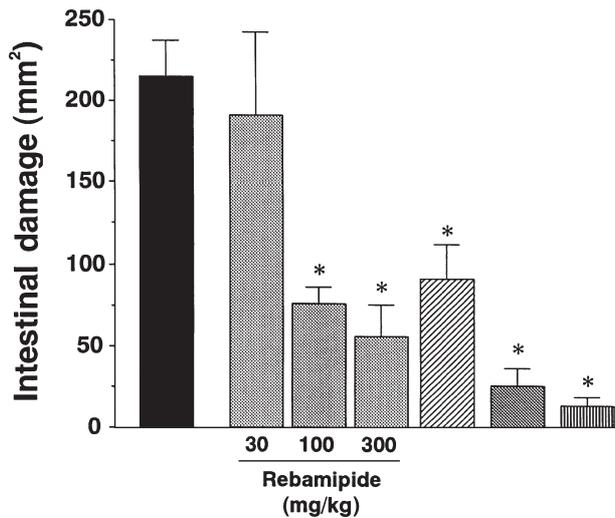
Figure 1 Effects of rebamipide and superoxide dismutase (SOD) + catalase (CAT) on the development of intestinal lesions induced by indomethacin in rats. Animals were given indomethacin s.c. in a dose of 10 mg/kg, killed 24 h later, and the area (mm^2) of lesions was measured under a dissecting microscope. Rebamipide (30–300 mg/kg, p.o.), SOD + CAT (3000 U + 5000 U/kg, i.v.) and 16,16-dimethyl prostaglandin E$_2$ (dmPGE2; 10 μg/kg, i.v.) was given twice, 30 min before and 6 h after indomethacin, while ampicillin (800 mg/kg, p.o.) was given twice 24 h and 30 min before indomethacin treatment. Data are presented as the mean ± SE from four to six (*n*) rats. ★Statistically significant difference from control at *P* < 0.05. (▨) Rebamipide, (■) control, (▧) SOD + CAT, (▥) ampicillin, (□) dmPGE$_2$.

Figure 2 Effect of rebamipide on the increase of myeloperoxidase (MPO) activity induced by indomethacin in rats. Animals were given indomethacin s.c. in a dose of 10 mg/kg and killed 24 h later. Rebamipide (100 mg/kg, p.o.) and superoxide dismutase (SOD) + catalase (CAT; 3000 U + 5000 U/kg, i.v.) were given twice, 30 min before and 6 h after indomethacin. Data are presented as the mean ± SE from six to seven (*n*) rats. Statistically significant difference at *P* < 0.05; [†]from normal; ★from the control.

elevated in response to indomethacin, reaching 0.20 ± 0.01 μmol H$_2$O$_2$/mg protein 24 h later (Fig. 2). The increased MPO activity was significantly suppressed by the treatment of the animals with either rebamipide (100 mg/kg) or SOD (3000 U/kg) + CAT (5000 U/kg), the values being 0.07 ± 0.01, 0.08 ± 0.02 μmol H$_2$O$_2$/min per mg protein, respectively. Likewise, both dmPGE$_2$ and ampicillin significantly prevented the increase of MPO activity following indomethacin (Fig. 3).

In contrast, the iNOS activity in the normal intestinal mucosa was 0.33 ± 0.12 pmol/min per mg protein (Fig. 4). Subcutaneous administration of indomethacin markedly increased iNOS activity in the intestinal mucosa when determined 24 h later, reaching approximately fourfold greater than basal levels, the values being 1.44 ± 0.21 pmol/min per mg protein. The increased iNOS activity following indomethacin was significantly reduced by pretreatment of the animals with rebamipide, dmPGE$_2$ or ampicillin at the doses that significantly prevented the occurrence of intestinal lesions, the inhibition being 43.1, 81.9 or 88.2%, respectively (Figs 3,4). Superoxidase dismutase + CAT tended to decrease the iNOS activity, but this effect was not significant when compared to control. Neither of these agents had any effect on cNOS activity in the intestinal mucosa.
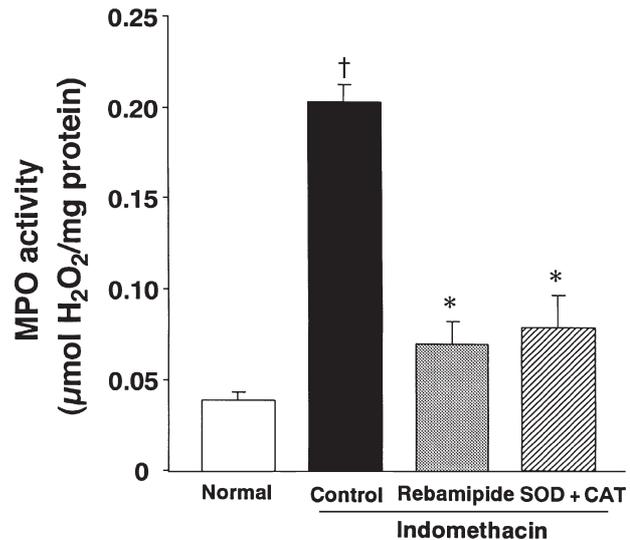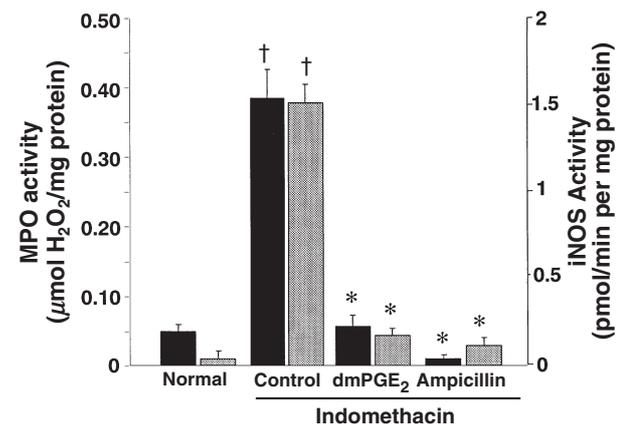

Figure 3 Changes in (■) myeloperoxidase (MPO) activity and (▨) inducible nitric oxide synthase (iNOS) activity in the rat small intestine after the administration of indomethacin, and the effects of 16,16-dimethyl prostaglandin E$_2$ (dmPGE$_2$) and ampicillin on these changes. Animals were given indomethacin (10 mg/kg) s.c., killed 24 h later, and the MPO activity was measured. dmPGE$_2$ (10 μg/kg) was given i.v. twice, 10 min before and 6 h after 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 from five to eight (*n*) rats. Significant difference at *P* < 0.05; ★from control; [†]from normal.
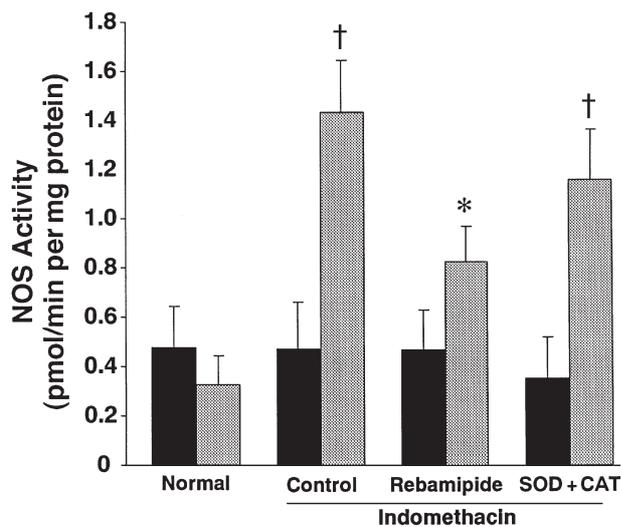
Figure 4 Changes of the nitric oxide synthase (NOS) activity in the small intestinal mucosa induced by indomethacin. Indomethacin (10 mg/kg) was administered s.c., and the animals were killed 24 h later. Rebamipide (100 mg/kg, p.o.) and superoxide dismutase (SOD) + catalase (CAT; 3000 U + 5000 U/kg, i.v.) were given twice 30 min before and 6 h after indomethacin treatment. Data are presented as the mean ± SE from five to seven (*n*) rats. Statistically significant difference at $P < 0.05$; [†]from normal; ★from control. (■) constitutive NOS, (▨) inducible NOS.

Subcutaneous administration of indomethacin also significantly increased the mucosal levels of lipid peroxidation as determined by TBA reactants in the small intestine 24 h later, the value being 0.53 ± 0.03 nmol/mg protein, which is approximately 1.7-fold greater than normal levels (Fig. 5). This increase in TBA reactants induced by indomethacin was all but completely inhibited when the animals were pretreated with either rebamipide or SOD + CAT. Similar effects were also observed by pretreatment with either dmPGE$_2$ or ampicillin (not shown).

Effect of rebamipide on enterobacterial translocation in small intestine following administration of indomethacin

The aerobic and anaerobic enterobacterial numbers in the normal intestinal mucosa were 7.16 ± 0.14 log CFU/g tissue and 7.04 ± 0.21 log CFU/g tissue, respectively (Fig. 6). Following subcutaneous administration of indomethacin (10 mg/kg), the enterobacterial numbers in both aerobic and anaerobic conditions were markedly increased to approximately 900-fold greater than controls, the values being 9.99 ± 0.05 log CFU/g tissue and 9.89 ± 0.03 log CFU/g tissue, respectively. Rebamipide (100 mg/kg) significantly prevented the increase of bacterial numbers in the mucosa following indomethacin. Likewise, the bacterial translocation in indomethacin-treated animals was slightly but significantly prevented by treatment of the animals with SOD
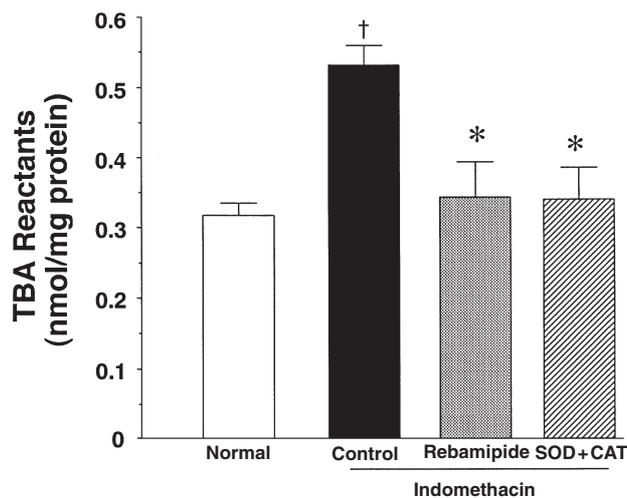

Figure 5 Effects of rebamipide and superoxide dismutase (SOD) + catalase (CAT) on the increase of thiobarbituric acid (TBA) reactants induced by indomethacin in normal rats. Animals were given indomethacin s.c. in a dose of 10 mg/kg and killed 24 h later. Rebamipide (100 mg/kg, p.o.) or SOD + CAT (3000 U + 5000 U/kg, i.v.) was given twice, 30 min before and 6 h after indomethacin. Data are presented as the mean ± SE from six to eight (*n*) rats. Statistically significant difference at $P < 0.05$; [†]from normal; ★from control.
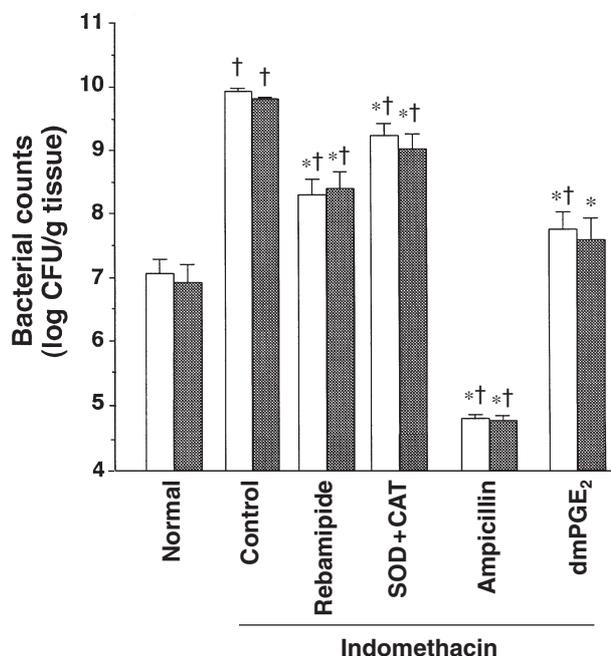

Figure 6 Changes in enterobacterial numbers invaded in the small intestinal mucosa induced by indomethacin. Indomethacin (10 mg/kg) was administered s.c., and the animals were killed 24 h later. Rebamipide (100 mg/kg, p.o.) and SOD + CAT (3000 U + 5000 U/kg, i.v.) were given twice 30 min before and 6 h after indomethacin treatment. Data are presented as the mean ± SE from six (*n*) rats. Statistically significant difference at $P < 0.05$; [†]from normal; ★from control. (□) Aerobic, (▨) anaerobic.

(3000 U/kg) + CAT (5000 U/kg). Certainly, both $dmPGE_2$ (10 µg/kg.) and ampicillin (800 mg/kg) also significantly reduced the bacterial translocation induced by indomethacin, and especially in the latter the bacterial number decreased to even lower than controls. Either of these agents was similarly effective in reducing the number in both aerobic and anaerobic bacterium.

DISCUSSION

The present study showed that rebamipide, a novel anti-ulcer drug with the radical scavenging property,[13,14,20,21] protected the intestinal mucosa from indomethacin-induced damage in rats. This protective effect was accompanied by the suppression of various inflammatory changes such as an increase of iNOS activity, MPO activity and lipid peroxidation. Similar effects were also observed by the combined treatment with SOD + CAT, suggesting the involvement of oxygen radicals in the pathogenesis of indomethacin-induced intestinal damage. Considering these findings, it is assumed that rebamipide exhibits a prophylactic effect against NSAID-induced intestinal damage, mainly because of its scavenging action.

First, we confirmed in the present study that supplementation with $dmPGE_2$ prevented not only the development of intestinal lesions in response to indomethacin, but also other changes in the mucosa such as increases in MPO and iNOS activities.[10,12,23] As expected, rebamipide exhibited a prophylactic action against these lesions, similar to $dmPGE_2$. Although multiple factors are considered in the pathogenic mechanism of indomethacin-induced intestinal damage,[5–9] recent studies suggested the importance of iNOS/NO as the pathogenic element, in addition to a PG deficiency.[9–11,26] Indeed, the prevention of these lesions was observed by a blockade of NO production by means of inhibiting the iNOS activity by N^G-nitro-L-arginine methyl ester or the iNOS expression by dexamethasone.[9,26,27] It is believed that NO interacts with the superoxide radicals to produce a cytotoxic peroxynitrite, which causes a deleterious influence on the intestinal mucosal integrity.[28,29] We previously reported that the severity of indomethacin-induced intestinal lesions was significantly reduced by inhibiting xanthine oxidase by allopurinol or reducing the number of neutrophils by hydroxyurea.[10] In the present study, we also observed that indomethacin-induced intestinal lesions were significantly prevented by the combined treatment with SOD + CAT. These results suggest that a detrimental role of NO in indomethacin-induced small intestinal lesions may be explained by a cytotoxic effect of peroxynitrite produced from NO in the presence of superoxide radicals. As rebamipide is known to have a potent scavenging action against superoxide radicals,[19–21] it is assumed that the protective effect of this drug is accounted for, at least partly, by its scavenging property.

In the present study, rebamipide also showed a significant suppression against the increase of MPO activity and iNOS activity as well as TBA reactants. This agent has been reported to inhibit by itself the neutrophil activation[30] and the iNOS expression.[31] Thus, rebamipide might inhibit these events directly, leading to the suppression of the subsequent changes such as iNOS expression and MPO activities as well as the peroxynitrite formation, which resulted in the prevention of the intestinal damage following indomethacin. Interestingly, like rebamipide, treatment with SOD + CAT showed only a tendency of decrease in iNOS activity suppression without a statistical significance. These different effects between rebamipide and SOD + CAT suggest that the former effect was not totally explained by the radical scavenging property, but was due to other actions including the inhibition of iNOS expression.

It should be noted in the present study that both rebamipide and SOD + CAT exhibited a significant suppression of the enterobacterial translocation following indomethacin, although the latter effect was less pronounced than that of rebamipide. Because the endotoxin released from bacteria induces iNOS expression,[11] as well as neutrophil recruitment,[6,12] it might be possible that the protective effect of rebamipide is brought about by the suppression of the bacterial translocation. At present, the exact mechanism of how rebamipide prevented the bacterial translocation following indomethacin remains unknown. Because the damage in the small intestine may increase the invasion of enterobacteria through the disrupted mucosal barrier, it is possible that the suppression of bacterial translocation is simply caused by a decrease in the severity of intestinal lesions induced by indomethacin. The same might occur in the animals pretreated with SOD + CAT. In contrast, we have recently shown that the bacterial translocation following indomethacin is causally related to an enhanced intestinal motility, as well as a decrease in the intestinal mucus secretion.[32,33] Supplementation with $dmPGE_2$ increased the mucus secretion and prevented the bacterial translocation, resulting in the inhibition of intestinal lesions.[32] Other drugs such as capsaicin or lafutidine also increased the mucus secretion through stimulation of capsaicin-sensitive sensory neurons, and resulted in protecting the small intestine against indomethacin.[23] Rebamipide has been reported to stimulate the mucus secretion in the stomach by a PG-independent mechanism.[17] It may thus be possible that this agent somehow hampers the process of bacterial translocation in the mucosa following indomethacin. Certainly, this point should be verified by examining the effect of rebamipide on intestinal mucus secretion.

Given the above findings, it was concluded that rebamipide affords a preventative effect against these lesions, together with the suppression of various inflammatory responses in the mucosa. The exact mechanism by which rebamipide protects the small intestine against indomethacin remains unknown, yet it may be attributable mainly to its radical scavenging action and partly to a direct inhibition of iNOS expression or neutrophil activation, as well as other protective actions yet to be identified. Taking this all into account, it is assumed that rebamipide may be used as a prophylactic agent against NSAID-induced intestinal damage.

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