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Title Page

Lubiprostone Prevents NSAID-Induced Small Intestinal Damage by Suppressing the Expression of Inflammatory Mediators via EP4 Receptors

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Running Title Page

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Lubiprostone and NSAID-Induced Enteropathy

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e) Nonstandard abbreviations used:

Nonsteroidal anti-inflammatory drugs ---------- NSAIDs

prostaglandins ---------- PGs

prostaglandin E_2 ---------- PGE_2

cyclooxygenase ---------- COX

cystic fibrosis transmembrane regulator ---------- CFTR

Myeloperoxidase ---------- MPO

inducible nitric oxide ---------- iNOS

tumor necrosis factor α ---------- TNFα

reverse transcriptional polymerase chain reaction --------- RT-PCR

glyceraldehyde-3-phosphate dehydrogenase ---------- GAPDH

short-circuit current ---------- I_{sc}

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Abstract

Lubiprostone, a bicyclic fatty acid derived from prostaglandin E_1, has been used to treat chronic constipation and irritable bowel syndrome, and its mechanism of action has been attributed to the stimulation of intestinal fluid secretion via the activation of ClC-2/CFTR chloride channels. We examined the effects of lubiprostone on indomethacin-induced enteropathy and investigated the functional mechanisms involved, including its relationship with the EP4 receptor subtype. Male SD rats were administered indomethacin (10 mg/kg) p.o. and killed 24 h later to examine the hemorrhagic lesions that developed in the small intestine. Lubiprostone (0.01-1mg/kg) was administered p.o. in a single injection 30 min before the indomethacin treatment. Indomethacin markedly damaged the small intestine, accompanied by intestinal hypermotility, a decrease in mucus and fluid secretion, and an increase in enterobacterial invasion as well as the up-regulation of iNOS and TNFα mRNAs. Lubiprostone significantly reduced the severity of these lesions, with the concomitant suppression of the functional changes. The effects of lubiprostone on the intestinal lesions and functional alterations were significantly abrogated by the co-administration of AE3-208, a selective EP4 antagonist, but not by CFTR(inh)-172, a CFTR inhibitor. These results suggested that lubiprostone may prevent indomethacin-induced enteropathy via an EP4 receptor-dependent mechanism. This effect may be functionally associated with the inhibition of intestinal hypermotility and increase in mucus/fluid secretion, resulting in the suppression of bacterial invasion and iNOS/TNFα expression, which are major pathogenic events in enteropathy. The direct activation of CFTR/ClC-2 chloride channels is unlikely to have contributed to the protective effects of lubiprostone.

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Introduction

Both short-term and long-term administration of nonsteroidal antiinflammatory drugs (NSAIDs) has been shown to cause intestinal ulceration in humans and laboratory animals (Fang et al., 1977; Whittle, 1981). Previous clinical studies using capsule endoscopes or double-balloon endoscopes confirmed that NSAIDs damaged the small intestine in more patients than previously thought (Goldstein et al., 2005; Maiden et al., 2005). However, except for the administration of prostaglandin (PG) analogs no satisfactory means are currently available to prevent and treat these lesions (Fujimori et al., 2009). This situation has been markedly intensified by recent findings in which drugs inhibiting acid secretion, such as proton pump inhibitors and histamine H_2 receptor antagonists, were ineffective against NSAID-induced small intestinal damage (Goldstein et al., 2005; Kato et al., 2000) and even worsened the severity of these lesions (Wallace et al., 2011; Satoh et al., 2012). However, they were shown to prevent the gastric ulcerogenic response to NSAIDs (Takeuchi et al., 1986; Satoh et al., 2012). Thus, the development of effective therapies to treat NSAID-induced small intestinal lesions remains an urgent priority.

Lubiprostone, a bicyclic fatty acid derived from PGE_1, has been used to treat chronic constipation and irritable bowel syndrome with constipation (Schey & Rao, 2011), and its mechanism of action has been attributed to the stimulation of intestinal fluid secretion via the activation of ClC-2 chloride channels which are located in the apical membranes of epithelial cells as a cystic fibrosis transmembrane regulator (CFTR) bypass channel in Cystic Fibrosis (Schwiebert et al., 1998; Cuppoletti et al., 2004). Previous studies have shown that this drug can activate PGE receptors (Bassil et al., 2008; Mizumori et al., 2009; Cuthbert, 2011). These receptors have been pharmacologically

subdivided into four subtypes, EP1-EP4 (Woodward et al., 2011). Among them, the EP4 receptor appears to be the main target for lubiprostone. Cuthbert (2011) demonstrated that EP4 receptors in sheep were the major target for lubiprostone to stimulate the secretion of anions in ovine airways. In addition, several studies showed that lubiprostone activated ClC-2/CFTR chloride channels via EP4 receptors (Cuppoletti et al., 2004; Bao et al., 2008; Bijvelds et al., 2009). We previously reported that PGE_2 ameliorated indomethacin-induced small intestinal damage via the activation of EP4 receptors (Kunikata et al., 2002a; Hatazawa et al., 2006; Takeuchi et al., 2012). Thus, lubiprostone may suppress the development of small intestinal lesions induced by NSAIDs by activating EP4 receptors.

In the present study, we examined the effects of lubiprostone on the intestinal ulcerogenic response induced by indomethacin in the rat and investigated the mechanisms responsible for its protective effects. Since lubiprostone is a derivative of PGE_1, we also examined whether its effects were mediated by prostanoid EP receptors, including the determination of EP receptor subtypes. In addition, we determined the contribution of ClC-2/CFTR channels to its protective effect in the small intestine.

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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±1ºC). Experiments were performedusing 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 Indomethacin

Animals were administered indomethacin (10 mg/kg) p.o. and euthanized 24 h later under deep ether anesthesia. The small intestine was excised, the tissue walls were fixed with 2% formalin for 10 min, and an opening was made along the anti-mesenteric attachment. The area of macroscopically visible damage (mm^2) was measured under a dissecting microscope with square grids (x10), summed per tissue, and used as a lesion score. A 1% Evans blue solution was administered i.v. in a volume of 1 ml/animal 30 min before animals were killed to highlight hemorrhagic lesions. The researcher measuring the lesions was blinded to the treatment given to the animals. Lubiprostone (0.01-1 mg/kg) was administered p.o. twice 30 min before and 9 h after the administration of indomethacin. In some cases, various EP antagonists such as ONO-8711 (an EP1 antagonist: 10 mg/kg)(Ukawa et al., 2002), AE-5-599 (an EP3 antagonist: 10 mg/kg)(Aihara et al., 2007) or AE3-208 (an EP4 antagonist: 3 mg/kg)(Hatazawa et al., 2006), and CFTR(inh)-172 (a CFTR inhibitor; 1 and 10 mg/kg)(Norimatsu et al., 2012) were administered i.p. 30 min before the administration

of lubiprostone. The doses of these EP and CFTR antagonists were chosen to induce the respective pharmacological action, according to the findings of previous studies (Ukawa et al., 2002; Kunikata et al., 2002a; Hatazawa et al., 2006; Aihara et al., 2007; Takeuchi et al., 2011; Norimatsu et al., 2012).

In some cases, the intestinal mucosa was examined under a light microscope following the administration of indomethacin (10 mg/kg) alone or indomethacin plus lubiprostone (0.1 mg/kg) with or without AE3-208 (3 mg/kg). Animals were euthanized 24 h after the indomethacin treatment, and the small intestine was excised. 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 MPO Activity

Myeloperoxidase (MPO) activity was measured according to a modified method of Krawisz et al. (1984). In brief, animals were euthanized under deep ether anesthesia 24 h after the indomethacin treatment, and the small intestine was removed. After rinsing with cold saline, the whole of intestine was weighed and homogenized in 50 mM phosphate buffer containing 0.5% hexadecyltrimethyl- ammonium bromide (HTAB, pH 6.0; Sigma). Homogenized samples were subjected to freeze-thawing three times and centrifuged at 2000 g for 10 min at 4°C. 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. 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 a spectrophotometric assay (protein assay kit; Pierce, Rockford, IL), and MPO activity was obtained from the slope of the reaction curve, according to the following equation; Specific

activity (μmol H_2O_2/min/ mg protein) = (OD/min)/OD/μmol H_2O_2 x mg protein). Lubiprostone (0.1 mg/kg) was given p.o. twice 30 min before and 9 h after the administration of indomethacin, while AE3-208 (3 mg/kg) was given i.p. 30 min before the administration of lubiprostone.

Determination of Enterobacterial Counts

Enterobacteria were enumerated according to a method described by Deitch et al. (1989). Animals were killed under deep ether anesthesia 6 h after the indomethacin treatment (10 mg/kg, p.o.), and the small intestine was 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 h under aerobic conditions (BBL Gas Pack Pouch Anaerobic System; BD Biosciences, San Jose, CA). The number of enterobacteria was counted on plates containing 10 to 300 colony-forming units (CFU), and the data were expressed as log CFU per gram of tissue. Lubiprostone (0.1 mg/kg) was administered p.o. 30 min before the administration of indomethacin. In some cases, AE3-208 (3 mg/kg) was given i.p. 30 min before the administration of lubiprostone.

Determination of Gene Expression of iNOS, TNFα, ClC-2 Chloride Channel, and EP1-4 Receptors

The gene expression of the ClC-2 chloride channel as well as EP1-4 receptors, inducible nitric oxide synthase (iNOS), and tumor necrosis factor α (TNFα) was measured in the small intestinal mucosa by reverse transcriptional polymerase chain reaction

(RT-PCR). The small intestine was removed under deep ether anesthesia, and stored at -80ºC prior to use. Both iNOS and TNFα mRNAs were determined in animals 6 h after the administration of indomethacin (10 mg/kg, p.o.). In some cases, lubiprostone (10 mg/kg) was given p.o. 30 min before the administration of indomethacin, while AE3-208 (3 mg/kg) was given i.p. 30 min before lubiprostone. Total RNA was extracted from tissue samples using Sepasol RNA I (Nacalai Tesque, Kyoto, Japan). 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 the rat ClC-2 chloride channel, EP1-4 receptors, iNOS, TNFα, 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 Image J (version 1.39), and semiquantitative measurements of mRNA expression levels were presented as a ratio compared with GAPDH.

Determination of Mucus Secretion

The amount of mucus secreted in the small intestine was determined using periodic acid-Schiff (PAS) staining. Animals were killed under deep diethyl ether anesthesia 3 h after the administration of indomethacin (10 mg/kg, p.o.), and the small intestine was 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 performed according to the conventional method. Lubiprostone (0.1 mg/kg) was given p.o. 30 min before the administration of indomethacin, while AE3-208 (3 mg/kg) was given i.p. 30 min before lubiprostone.

Determination of Intestinal Motility

Intestinal motility was measured according to a modified version (Takeuchi et al., 2002) of the method originally reported by Calignano et al. (1992). In brief, rats fasted for 18 h 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). Indomethacin (10 mg/kg) was given s.c. after basal intestinal motility had stabilized, and motility was measured for 3 h thereafter. Lubiprostone (0.1 mg/kg) was given i.p. 2 h after the indomethacin treatment, while AE3-208 (3 mg/kg) was given i.p. 30 min before lubiprostone.

Determination of Enteropooling

The enteropooling assay was performed according to the method described by Robert et al (1976). Animals were deprived of food for 24 h, but allowed free access to

tap water until 1 h before the experiment. Animals were administered indomethacin (10 mg/kg) p.o., and were killed under deep ether anesthesia 3 h later. The abdomen was opened, and both the pylorus and end of the ileum were clamped immediately. The fluid that accumulated in the lumen of the small intestine for 3 h was then collected in a graduated tube and centrifuged for 10 min at 3000 rpm, and volume was measured to the nearest 0.01 ml. Lubiprostone (0.1 mg/kg) was given p.o. 30 min before the administration of indomethacin, while AE3-208 (3 mg/kg) was given i.p. 30 min before lubiprostone.

Measurement of I_{sc} in the rat small intestines

Animals were killed under deep diethyl ether anesthesia, and the ileum (2-4 cm proximal to the cecum) was removed. The ileal segment was flushed with cold saline and opened along the mesenteric attachment. Tissue sheets were mounted in an Ussing chamber (EM-CSYS-2, Physiologic Instruments, San Diego, CA) after blunt dissection of the outer muscle layers. The exposed area was 0.49 cm^2, and the tissue sheets were bathed in unbuffered saline (154 mM NaCl) gassed with 100% O_2 on the mucosal side and Krebs-Ringer's solution (mM: 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, 11 Glucose) gassed with 95% O_2, 5% CO_2 on the serosal side. These solutions were warmed at 37ºC and continuously circulated using a gas-lift system. The resulting short-circuit current (I_{sc}), reflecting transepithelial electrogenic ion transport, was recorded with a multi-channel recorder (U-228, Tokai-irika, Tokyo, Japan). Lubiprostone (0.1 nM-10 μM) was added to either the mucosal or serosal solution, and AE3-208 (1 μM) was applied 15 min before the treatment with lubiprostone.

Preparation of Drugs

The drugs used were indomethacin (Sigma Chemicals, St. Louis, MO), lubiprostone (Abbott Japan Co., Ltd. Tokyo, Japan), ONO-8711 (an EP1 antagonist), AE5-599 (an EP3 antagonist), AE3-208 (an EP4 antagonist) (Ono Pharmaceutical Co., Ltd., Osaka, Japan), CFTR(inh)-172 (a CFTR inhibitor; Wako Pure Chemicals, Osaka, Japan), and urethane (Tokyo Kasei, Tokyo, Japan). Indomethacin was suspended in a 0.5% hydroxypropylcellulose solution (Wako Pure Chemicals), while other drugs were 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. In some cases, lubiprostone with or without AE3-208 was added to either the mucosal or serosal solution in an isolated ileum preparation in a volume of 100 µl. Control animals received the vehicle alone.

Statistical Analyses

Data are presented as means±SE for four to eight 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.

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Results

Effect of Lubiprostone on Indomethacin-Induced Small Intestinal Damage

Indomethacin (10 mg/kg) given orally as a single injection produced multiple hemorrhagic lesions in the small intestine of normally fed rats, mainly in the jejunum and ileum, and the lesion score was 349±46.2 mm^2 **(Fig. 1)**. The pretreatment with lubiprostone (0.01-1 mg/kg), which given p.o. 30 min before and 9 h after indomethacin, dose-dependently prevented the development of these lesions in response to indomethacin. This effect was significant at 0.03 mg/kg or greater; the lesion score at 0.1 mg/kg was 16.0±4.8 mm^2, and inhibition was 95.4%. Based on these results, we used lubiprostone at 0.1 mg/kg in subsequent experiments.

The prior administration of lubiprostone (0.1 mg/kg) markedly reduced the severity of indomethacin-induced small intestinal damage. The protective effects of lubiprostone were significantly affected by the co-treatment with the selective EP4 antagonist, AE3-208 (3 mg/kg, i.p.); the lesion score was 272±64.2 mm^2, which was not significantly different from that of the control (349±46.2 mm^2) **(Fig. 2-I)**. However, neither the EP1 antagonist, ONO-8711 (10 mg/kg, i.p.), nor the EP3 antagonist, AE5-599 (10 mg/kg, i.p.), had any effect on the protective effects of lubiprostone. Histologically, indomethacin produced severe lesions in the small intestine, which deeply infiltrated the mucosa, almost reaching the muscularis mucosae **(Fig. 2-II)**. Lubiprostone (0.1 mg/kg) completely suppressed this damage in the mucosa following the indomethacin treatment, however this damage was severe in rats co-administered the EP4 antagonist, AE3-208 (3 mg/kg), with lubiprostone.

Effect of Lubiprostone on Various Events Induced in the Small Intestine by Indomethacin

Lubiprostone prevented the occurrence of damage in the small intestine after the indomethacin treatment, and this effect at 0.1 mg/kg was potent and reproducible. To further investigate the functional mechanisms responsible for the protective effects of lubiprostone, we examined the effects of this drug at 0.1 mg/kg on various events that are considered to be critical to the pathogenesis of NSAID-induced enteropathy (Takeuchi et al., 2010a).

MPO activity: MPO activity in the normal small intestine was 0.04±0.01 μmol H_2O_2/min/mg protein and markedly increased in response to indomethacin (10 mg/kg, p.o.), reaching 0.13±0.03 μmol H_2O_2/min/mg protein after 24 h. This increase in MPO activity was significantly suppressed by lubiprostone (0.1 mg/kg, p.o.), and was reduced to 0.03±0.01 μmol H_2O_2/min/mg protein. The inhibitory effect of lubiprostone was significantly abrogated by the pretreatment with the EP4 antagonist, AE3-208 (3 mg/kg, i.p.), to 0.13±0.02 μmol H_2O_2/min/mg protein.

Mucosal expression of iNOS and TNFα mRNAs: GAPDH mRNA, the housekeeping gene, was clearly detectable in the small intestines of control rats and its expression remained unaffected by the administration of indomethacin. The expression of iNOS mRNA was not detected in the normal intestine, but was markedly up-regulated (approximately10 times higher than that in the control) in mucosa examined 6 h after the p.o. administration of indomethacin (10 mg/kg) **(Figs. 3A and 3B)**. The pretreatment with lubiprostone (0.1 mg/kg, p.o.) appeared to suppress the indomethacin-induced

up-regulation of iNOS expression. TNFα mRNA expression was also very weak in the normal mucosa, but was markedly up-regulated after the administration of indomethacin. This up-regulation was also suppressed by the prior administration of lubiprostone. The suppressive effects of lubiprostone on the up-regulated expression of iNOS and TNFα mRNAs were completely reversed by the co-treatment with the EP4 antagonist, AE3-208 (3 mg/kg, i.p.).

Enterobacterial invasion: Aerobic and anaerobic bacterial counts in the intestinal mucosa of normal rats were 6.97±0.13 and 6.92±0.14 log CFU/g tissue, respectively. Those following the administration of indomethacin (10 mg/kg, p.o.) were approximately 10 times greater after 6 h, at 8.15±0.15 and 8.36±0.06 log CFU/g tissue, respectively **(Table 2)**. The enhanced invasion of enterobacteria following the indomethacin treatment was significantly prevented by the pretreatment with lubiprostone (0.1 mg/kg, p.o.), and this effect was totally alleviated by the prior administration of the EP4 antagonist, AE3-208 (3 mg/kg, i.p.).

Mucosal expression of PAS-positive materials: In the normal intestinal mucosa, PAS-positive materials were observed in surface epithelial cells and the lamina propria along the intestinal gland **(Fig. 4A)**. Indomethacin (10 mg/kg, p.o.) markedly reduced PAS-positive materials in the intestinal mucosa, particularly in surface epithelial cells **(Fig. 4B)**. The decrease in PAS-positive materials was largely restored by the pretreatment with lubiprostone (0.1 mg/kg, p.o.)**(Fig. 4C)**, but this effect was abrogated by the co-administration of the EP4 antagonist, AE3-208 (3 mg/kg. i.p.)**(Fig. 3D)**. The expression of PAS-positive materials was higher in lubiprostone-treated rats than in

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normal rats (data not shown).

Enteropooling: Control animals accumulated 0.56±0.04 ml of fluid in the small intestine for 3 h **(Fig. 5)**. The oral administration of indomethacin (10 mg/kg, p.o.) significantly decreased the amount of fluid in the small intestine to approximately 39% that of the control value. This effect of indomethacin was significantly reversed by the prior administration of lubiprostone (0.1 mg/kg, p.o.), with the amount of fluid being restored to 0.82±0.11 ml. The fluid-stimulating effect of lubiprostone was completely attenuated by the co-administration of AE3-208, the EP4 antagonist (3 mg/kg, i.p.). Lubiprostone by itself increased the luminal accumulation of fluid, to approximately 1.4-fold that in normal rats (not shown).

This action was also confirmed *in vitro* using isolated ileum preparations. The application of lubiprostone (0.1 nM-10 μM) to either the mucosal or serosal solution increased the net I_{sc} in a concentration-dependent manner **(Fig. 6)**. The stimulatory effects of lubiprostone under both conditions was significantly inhibited by the co-application of AE3-208 (1 μM).

Intestinal hypermotility: Under urethane anesthesia, indomethacin (10 mg/kg) given s.c. markedly enhanced intestinal motility, in terms of both the amplitude and frequency of contractions 20-30 min after its administration **(Fig. 7A)**. Lubiprostone (0.1 mg/kg, i.p.), given 2 h after the indomethacin treatment, potently suppressed the indomethacin-induced enhancement in motility **(Fig. 7B)**; however, this response was completely alleviated in the presence of the EP4 antagonist, AE3-208 (3 mg/kg, i.p.)**(Fig. 7C)**.

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Effect of CFTR(inh)-172 on the Protective Action of Lubiprostone Against Indomethacin-Induced Small Intestinal Damage

Since lubiprostone is known to induce its effects by activating ClC-2 chloride channels on the apical aspect of gastrointestinal epithelial cells to produce a chloride-rich fluid secretion, the direct activation of these channels by this drug may contribute to the intestinal protective effects of this drug. Therefore, we examined the influence of CFTR(inh)-172, the CFTR inhibitor, on the protective effects of lubiprostone against indomethacin-induced enteropathy.

The severity of indomethacin-induced intestinal lesions was significantly reduced by the prior administration of lubiprostone (0.1 mg/kg), with the inhibition being 91.6%. This protection was not significantly affected by the pretreatment with the CFTR inhibitor, CFTR(inh)-172 (1 and 10 mg/kg, i.p.); lubiprostone inhibited the severity of these lesions by over 85%, even in the presence of 10 mg/kg of CFTR(inh)-172 **(Fig. 8)**.

Gene Expression of ClC-2 Chloride Channel and EP1-EP4 Receptors in the Rat Small Intestine

Because EP4 receptors were found to be involved in the intestinal protective action of lubiprostone, we examined the gene expression of various EP receptor subtypes (EP1-4) as well as the ClC-2 chloride channel. As shown in **Figure 9**, EP1-4 receptors were expressed in the small intestinal mucosa (ileum), although differences were observed in the intensities of their expression. The gene expression of the ClC-2 chloride channel was also clearly detected in the small intestine.

Discussion

Lubiprostone, a bicyclic fatty acid derived from PGE_1, has been used to treat chronic constipation (Schey & Rao, 2011), and its mechanism of action has been attributed to the stimulation of intestinal fluid secretion via the activation of ClC-2 chloride channels located in the apical membranes of epithelial cells (Schwiebert et al., 1998; Cuppoletti et al., 2004). Previous studies reported the beneficial effects of prostanoids, especially the E type of PG, against NSAID-induced small intestinal damage in experimental animals (Kunikata et al., 2002a; Hatazawa et al., 2006; Takeuchi et al., 2010b; Satoh et al., 2014). Furthermore, since intestinal fluid secretion is thought to hamper enterobacterial invasion, the major pathogenic event in NSAID-induced enteropathy (Takeuchi et al., 2010a), the activation of ClC-2 chloride channels may alleviate the occurrence of intestinal damage by stimulating fluid secretion. Thus, we examined whether lubiprostone exhibited a protective effect against indomethacin-induced small intestinal damage mediated by prostanoid EP receptors and/or depending on the activation of ClC-2 chloride channels.

The present study demonstrated that lubiprostone significantly prevented the development of lesions in the small intestine in response to indomethacin. The development of these lesions is known to be accompanied by intestinal hypermotility, a decrease in mucus/fluid secretion, enterobacterial invasion, and the up-regulation of iNOS expression, events that play a critical role in the pathogenesis of these lesions (Takeuchi et al., 2002; 2006; 2010a). We also previously reported that a selective cyclooxygenase (COX)-1 inhibitor did not provoke any gross damage in the small intestine because it up-regulated the expression of COX-2, while COX-2 derived PGE_2 suppressed the deleterious events caused by the inhibition of COX-1 (Tanaka et al., 2002). Since

lubiprostone is derived from PGE_1 and induces its pharmacological effects through EP receptors (Bassil et al., 2008; Mizumori et al., 2009; Cuthbert, 2011), it is possible that this drug protects the small intestine against NSAIDs even under PGE_2-deficient conditions. As expected, lubiprostone prevented indomethacin- induced lesions in the small intestine, and this effect was significantly abrogated by the co-administration of AE3-208, the EP4 antagonist, which suggested the involvement of EP4 receptors in the protective effects. These results are consistent with our previous findings in which PGE_2 protected the small intestine against NSAID mediated by EP4 receptors (Kunikata et al., 2002a; Takeuchi et al., 2007; Takeuchi et al., 2010a). Lubiprostone is unlikely to have prevented intestinal damage by increasing endogenous PG levels. These findings also suggest that lubiprostone may act downstream of the events resulting from PG deficiency caused by indomethacin and eventually prevents the development of small intestinal lesions.

Robert et al. (1977) reported that germ-free rats were resistant to indomethacin-induced intestinal damage, which indicated the key pathogenic role of enterobacteria in this model. These intestinal lesions were prevented by a pretreatment with antibiotics such as ampicillin (Tanaka et al., 1999; Takeuchi et al., 2006). Boughton-Smith et al. (1993) demonstrated that bacterial endotoxin enhanced intestinal permeability by up-regulating iNOS and the overproduction of NO in the mucosa. This hypothesis was supported by indomethacin increasing iNOS activity and NO production, preceeding the onset of intestinal damage, and aminoguanidine mitigating the intestinal ulcerogenic response by suppressing NO production due to iNOS (Tanaka et al., 1999; Takeuchi et al., 2006). In the present study, we also demonstrated that indomethacin increased bacterial invasion in the mucosa, followed by the up-regulation of iNOS expression and MPO activity, and these responses were also suppressed by lubiprostone. These results suggest that the protective effect of lubiprostone against

indomethacin-induced intestinal damage may be functionally associated with the down-regulation of iNOS expression and neutrophil recruitment due to the suppression of bacterial invasion. The proinflammatory cytokine TNFα is also capable of inducing iNOS expression in various cells. Bertrand et al. (1998) demonstrated that NSAIDs induced the local production of TNFα in the small intestine and this occurred prior to the elevation in NO production and MPO activity as well as lesion formation. We confirmed the increased expression of TNFα mRNA in the small intestine following the indomethacin treatment in the present study and found that lubiprostone appeared to suppress this increase, in an EP4 receptor-dependent manner. Thus, the protective effect of lubiprostone may be accounted for, at least partly, by the suppression of inflammatory mediators such as TNFα and iNOS following the indomethacin treatment.

The mechanism by which enterobacteria invade the mucosa remains unknown; however, previous studies have suggested that a decrease in mucus secretion may contribute to this process following the administration of NSAIDs (Kunikata et al., 2002a; Kamei et al., 2008; Amagase et al., 2010; Hayashi et al., 2013). Because mucus plays a crucial role in innate host defenses against intestinal pathogens and irritants, it is possible that a decrease in mucus secretion may weaken the intestinal barrier, resulting in bacterial invasion. The expression of PAS-positive materials in the small intestine was markedly reduced after the indomethacin treatment in the present study, and lubiprostone restored the decrease in PAS-positive materials caused by indomethacin in an EP4 receptor-dependent manner. It is assumed that lubiprostone up-regulates mucus secretion, thereby increasing the thickness of mucus gel and hampering bacterial invasion after the indomethacin treatment. Likewise, intestinal fluid secretion has been shown to prevent bacterial invasion by washing out the invading bacteria (Kunikata et al., 2002a; Hayashi et al, 2013). Indomethacin decreased the amount of fluid that

accumulated in the small intestine, and this response was reversed by the prior administration of lubiprostone, in an EP4 antagonist-inhibitable manner. We also showed that lubiprostone increased I_{sc}, which reflected Cl^- secretion, in isolated ileum preparations, and this action was alleviated by the EP4 antagonist, AE3-208. Since the stimulatory effect of fluid secretion was previously shown to be caused by the activation of ClC-2 chloride channels (Cuppoletti et al., 2004; Schey & Rao, 2011), the increase in fluid secretion may be partly attributed to the activation of these channels. However, the protective effects of lubiprostone were not significantly mitigated by the co-administration of CFTR(inh)-172, the inhibitor of CFTR, which suggests that the direct activation of CFTR/ClC-2 chloride channels did not contribute to the protective effects of lubiprostone against indomethaicn-induced intestinal damage. We confirmed the gene expression of EP1-EP4 receptors as well as ClC-2 chloride channels in the rat ileum in the present study; however, the cell type that expresses each EP receptor subtype and ClC-2 chloride channels remains unknown.

Intestinal hypermotility is also considered to play a role in the pathogenesis of NSAID-induced small intestinal damage (Takeuchi et al., 2002; 2010a; Kunikata et al., 2002b; Satoh et al., 2012). The abnormal hypermotility caused by NSAIDs may disrupt the unstirred mucus layer over the epithelium, leading to the expedition of enterobacterial invasion. We confirmed that indomethacin enhanced intestinal motility in this study, and this response occurred within 20-30 min of its administration, which was markedly earlier than the onset of other pathogenic events such as bacterial invasion, neutrophil activation, and iNOS expression. PGE_2 is known to suppress the intestinal hypermotility response to indomethacin by activating EP4 receptors (Kunikata et al., 2002a). Lubiprostone also inhibited the hypermotility response and this effect was abrogated by the EP4 antagonist. These results suggest that the anti- hypermotility effect of

lubiprostone may be mediated by EP4 receptors and further show the participation of this effect in the protective mechanism against intestinal damage.

The results of the present study indicate that lubiprostone prevented indomethacin-induced enteropathy via an EP4 receptor-dependent mechanism, and this effect may be functionally associated with the inhibition of intestinal hypermotility and increase in mucus/fluid secretion, resulting in the suppression of bacterial invasion and iNOS/TNFα expression, major pathogenic events in the enteropathy. In addition, the direct activation of CFTR/ ClC-2 chloride channels is unlikely to have contributed to the protective effects of lubiprostone on NSAID-induced small intestinal damage. These results suggest that lubiprostone may be used as a prophylactic agent against NSAID-induced small intestinal damage.

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Authorship Contributions

Participated in the research design: SH and KT

Conducted experiments: SH, NK, KA, and AY

Contributed new reagents or analytic tools: None

Performed data analysis: SH, NK, KA, and KT

Wrote or contributed to the writing of the manuscript: SH and KT

Other: None

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Footnotes:

a) Finacial support: We did not have any financial support for this study. We also have no conflicts of interest.

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d) Numbered footnotes: None

Legends for Figures

Figure 1. Effect of lubiprostone on the development of intestinal lesions induced by indomethacin in rats. Animals were given indomethacin p.o. in a dose of 10 mg/kg and killed 24 h later. Lubiprostone (0.01-1 mg/kg) was given p.o. twice 30 min before and 9 h after the administration of indomethacin. Data are presented as means±SE from 6 rats. * Significant difference from the control, at $P<0.05$.

Figure 2. Effect of various EP receptor antagonists on the protective action of lubiprostone against intestinal damage induced by indomethacin in rats. Animals were given indomethacin p.o. in a dose of 10 mg/kg and killed 24 h later. Lubiprostone (0.1 mg/kg) was given p.o. twice 30 min before and 9 h after the administration of indomethacin. EP1 antagonist (ONO-8711: 10 mg/kg), EP3 antagonist (AE5-599: 3 mg/kg) or EP4 antagonist (AE3-208: 3 mg/kg) was given i.p. 30 min before the administration of lubiprostone. Data are presented as means±SE from 5-6 rats. Significant difference at $P<0.05$; * from the control; # from the vehicle. Figure II shows microscopic observations of indomethacin-induced small intestinal lesions in rats (x40); A: Normal; B: Indomethacin alone; C: Lubiprostone + Indomethacin; D: AE3-208 + Lubiprostone + Indomethacin.

Figure 3. Effect of lubiprostone on the expression of iNOS and TNFα mRNA in the rat small intestine after the indomethacin treatment. Animals were administered indomethacin (10 mg/kg) p.o. and killed 6 h later to examine the expression of iNOS and TNFα mRNAs by RT-PCR. In the combined administration, lubiprostone (0.1 mg/kg) was given p.o. 30 min before the administration of indomethacin, while AE3-208 (3

mg/kg) was given i.p. 30 min before lubiprostone. M: Marker. In Figure B, densitometric quantitation was performed with Image J software, and the results are expressed as the ratio of iNOS or TNFα to GAPDH. Data are presented as means±SE for 4 rats. Significant differences at $P<0.05$; * from Normal; # from the control (indomethaicn alone). Indomethacin up-regulated both iNOS and TNFα expression; however, these responses appeared to be suppressed by the prior administration of lubiprostone in an EP4 receptor- dependent manner.

Figure 4. Effect of lubiprostone on changes in PAS-positive substances caused by indomethacin in the rat small intestine. Animals were administered indomethacin (IM: 10 mg/kg, s.c.) and sacrificed 6 h later. Lubiprostone (Lubi: 0.1 mg/kg) was administrated p.o. 30 min before the indomethacin treatment. AE3-208 (AE: 3 mg/kg) was given i.p. 30 min before the administration of lubiprostone.

Figure 5. Effects of lubiprostone on enteropooling (fluid secretion) in the rat small intestine, with or without the indomethacin treatment. Animals were administered lubiprostone (0.1 mg/kg) or indomethacin (10 mg/kg) p.o., either alone or in combination, and the amount of fluid that accumulated in the small intestine was measured 3 h later. In the combined administration, lubiprostone was given p.o. 30 min before indomethacin, while AE3-208 (3 mg/kg) was given i.p. 30 min before lubiprostone. Data are presented as means±SE for 5-6 rats. Significant differences at $P < 0.05$: * from normal; # from the control; $ from the vehicle.

Figure 6. Effect of the EP4 antagonist on the cumulative concentration-response curve for lubiprostone-induced short-circuit current (Isc) in the rat small intestine.

Lubiprostone (0.1 nM-10 μM) was applied to the mucosal or serosal side compartment of the Ussing chamber. AE3-208 (1 μM) was applied to the serosal side 15 min before the treatment with lubiprostone. Data are presented as means±S.E. from 4-6 rats. * Significant differences from the vehicle, at $p<0.05$.

Figure 7. Representative recordings showing the effect of lubiprostone on the enhanced small intestinal motility caused by indomethacin in urethane-anesthetized rats. Indomethacin (10 mg/kg) was given s.c., and small intestinal motility was recorded for 3 h thereafter. Lubiprostone (0.1 mg/kg) was given i.p. 2 h after the administration of indomethacin. The EP4 antagonist AE3-208 (3 mg/kg) was given i.p. 30 min before lubiprostone. Note that lubiprostone inhibited the intestinal hypermotility caused by indomethacin, and this effect was completely attenuated in the presence of AE3-208.

Figure 8. Effect of CFTR(inh)-172 on the suppressive action of lubiprostone for the development of small intestinal lesions induced by indomethacin in rats. Animals were given indomethacin (10 mg/kg, s.c.) and killed 24 h later. Lubiprostone (0.1 mg/kg) was given p.o. twice 30 min before and 9 h after the administration of indomethacin. CFTR(inh)-172 (CFTR inhibitor; 1-10 mg/kg) was given i.p. 30 min before the administration of lubiprostone. Data are presented as means±SE from 4-5 rats. Significant differences at $P<0.05$, * from indomethacin.

Figure 9. Expression of ClC-2 and EP1-EP4 receptor mRNAs in the rat small intestine. Bands from left showed ClC-2 chloride channels (N=2), EP1 receptors (N=2), EP2 receptors (N=2), EP3 receptors (N=2), and EP4 receptors (N=2). M: marker

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Gene	Primer Sequence 5'-3'	PCR Product
ClC2		499 bp
Forward	CAAGTTCCTCTCCCTCTTTG	
Reverse	GAACTGTCCAAAGCCAGGG	
EP1		778 bp
Forward	CCCAGGGTCCCCAATACATCT	
Reverse	GGGCAGCTGTGGTTGAAG	
EP2		1178 bp
Forward	CGCCCTCCACCATGGACAAT	
Reverse	AAGCAGCGCATGCTCACAAC	
EP3		666 bp
Forward	TGGCTGGCGCTCACCGACTTG	
Reverse	GCATTGCTCTACTGACATCTG	
EP4		488 bp
Forward	CCCTGCAGCGCCTCAGTGACTTT	
Reverse	CTTGCCTCCGAGGCTGCTTTCAGT	
TNF-α		331 bp
Forward	GTCCCAACAAGGAGGAGAAGTT	
Reverse	TCCTGGTATGAAGTGGCAAATC	
iNOS		780 bp
Forward	CGGTTCACAGTCTTGGTGAAAG	
Reverse	CAGGTGTTCCCCAGGTAGGTAG	
GAPDH		331 bp
Forward	GAACGGGAAGCTCACTGGCATGGC	
Reverse	TGAGGTCCACCACCCTGTTGCTG	

Table 1. Sequences of the sense and antisense primers for rat ClC-2, EP1~EP4 receptors, iNOS, and TNFα.

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Treatment	Number of Rats	Enterobacterial Number (log CFU/g tissue)	
		Aerobic	Anaerobic
Normal	7	6.57±0.27	6.58±0.26
Indomethacin	8	8.35±0.28*	8.49±0.20*
+ Lubiprostone	8	5.72±0.22$	6.75±0.15$
+ AE3-208	7	7.97±0.24#	7.90±0.30#

Table 2. Effect of lubiprostone on indomethacin-induced increase in bacterial invasion in rat small intestinal mucosa. Animals were administered saline or indomethacin (10 mg/kg, s.c.) and killed 24 h later. Lubiprosotone (0.1 mg/kg) was given p.o. twice 30 min before and 9 h after the administration of indomethacin. The EP4 antagonist (AE3-208: 3 mg/kg) was given i.p. 30 min before the administration of lubiprostone. Data are presented as means±SE from 7-8 rats. Significant differences at $P<0.05$; * from normal, $ from indomethacin alone, # from lubiprostone+indomethacin.

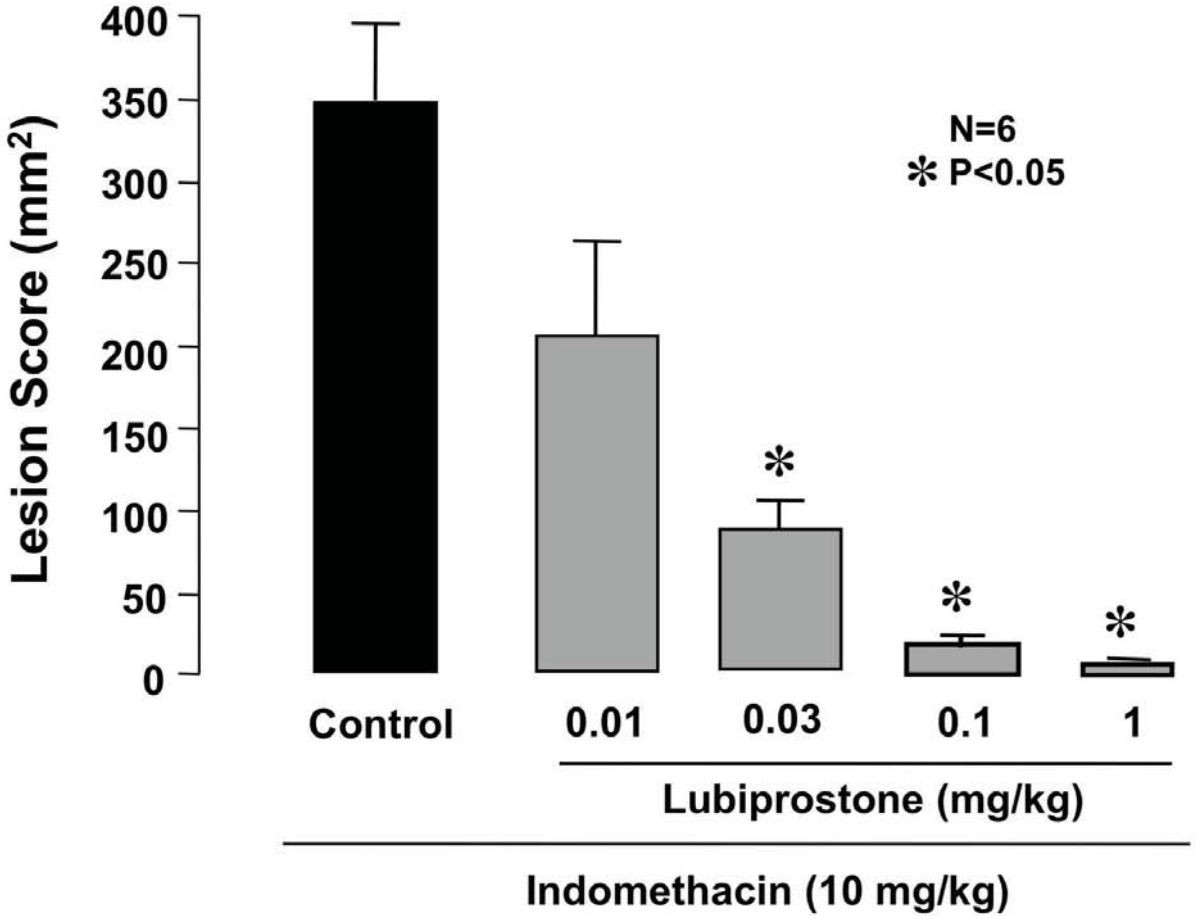

Figure 1

Figure 2

Figure 3

Figure 4

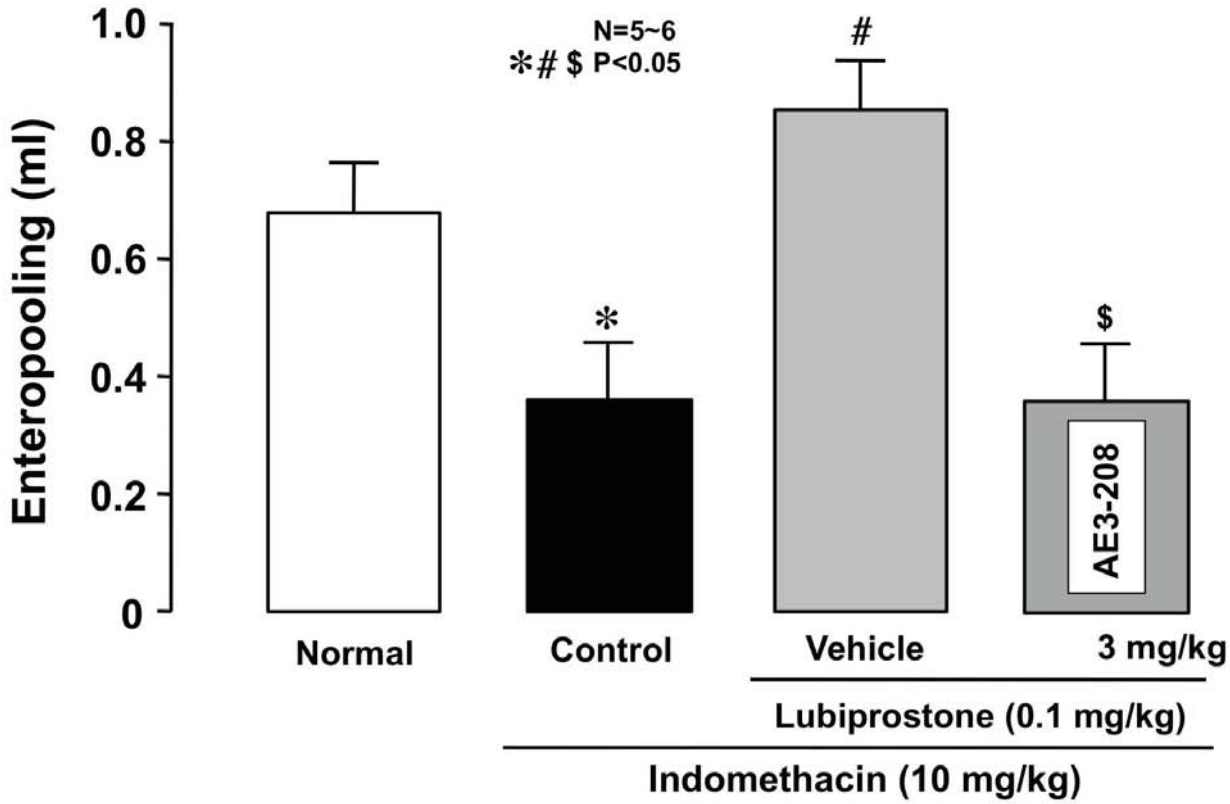

Figure 5

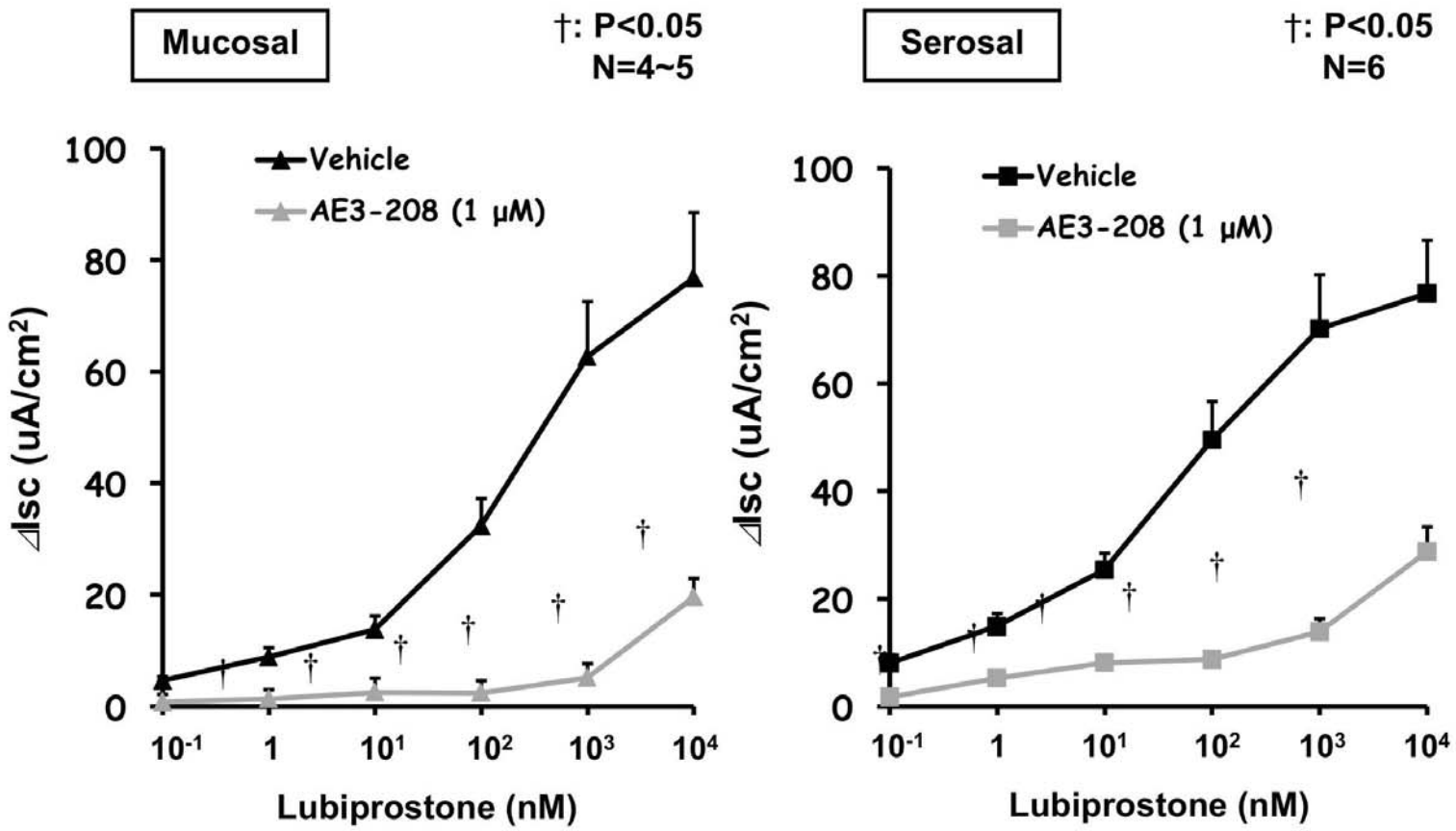

Figure 6

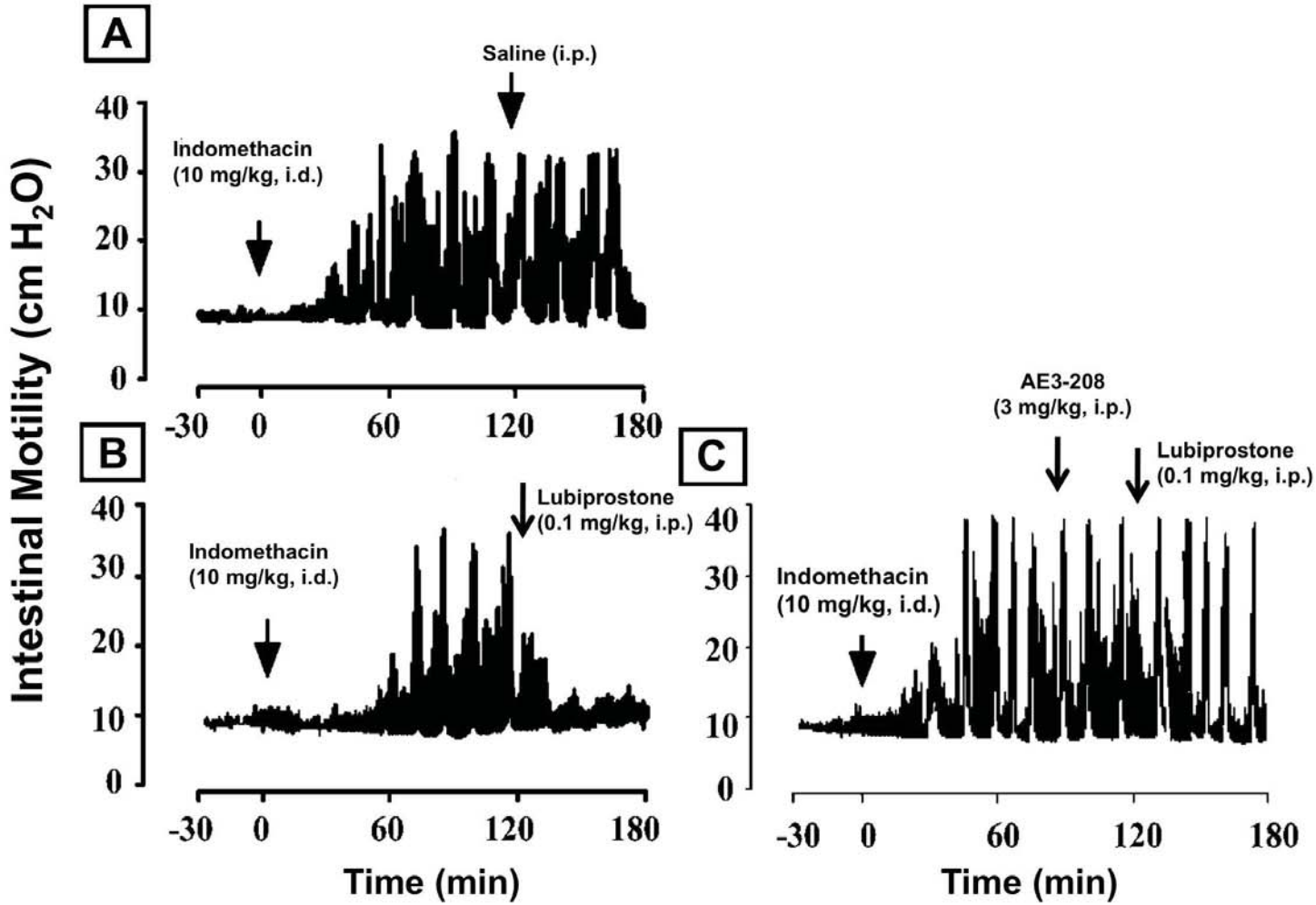

Figure 7

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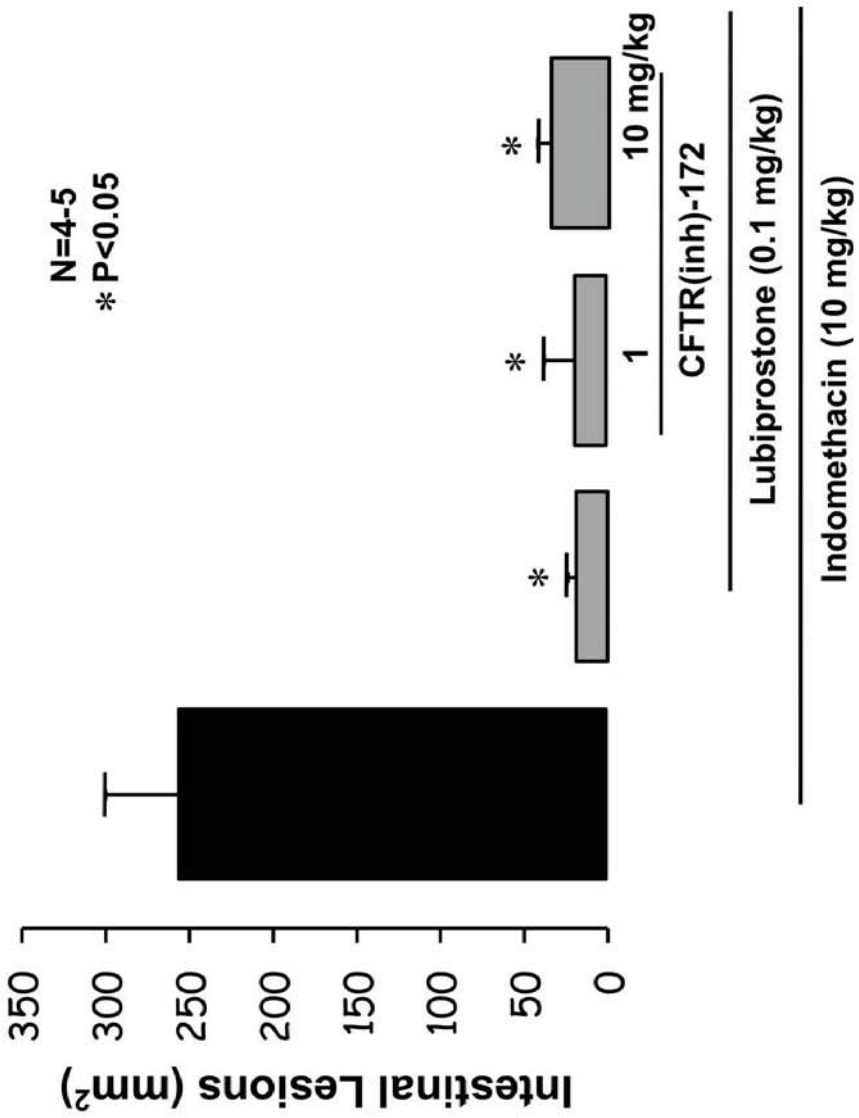

Figure 8

Figure 9