EP4 agonist alleviates indomethacin-induced gastric lesions and promotes chronic gastric ulcer healing

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Abstract

AIM: To investigate EP4-selective agonist effect on indomethacin-induced gastric lesions and on the spontaneous healing of chronic gastric ulcers.

METHODS: In a mouse model of gastric bleeding with high dose of indomethacin (20 mg/kg), an EP4-selective agonist was administered orally. Stomach lesions and gastric mucous regeneration were monitored. In a mouse model of chronic gastric ulcer induced by acetic acid, EP4 agonist effect on the healing of chronic gastric ulcer was evaluated in the presence or absence of low dose indomethacin (3 mg/kg). In cultured human gastric mucous cells, EP4 agonist effect on indomethacin-induced apoptosis was assessed by flow cytometry.

RESULTS: The EP4-selective agonist reduced high dose indomethacin-induced acute hemorrhagic damage and promoted mucous epithelial regeneration. Low-dose indomethacin aggravated ulcer bleeding and inflammation, and delayed the healing of the established chronic gastric ulcer. The EP4 agonist, when applied locally, not only offset indomethacin-induced gastric bleeding and inflammation, but also accelerated ulcer healing. In the absence of indomethacin, the EP4 agonist even accelerated chronic gastric ulcer healing and suppressed inflammatory cell infiltration in the granulation tissue. In vitro, the EP4 agonist protected human gastric mucous cells from indomethacin-induced apoptosis.

CONCLUSION: EP4-selective agonist may prevent indomethacin-induced gastric lesions and promote healing of existing and indomethacin-aggravated gastric ulcers, via promoting proliferation and survival of mucous epithelial cells.

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Key words: Prostaglandin E2; Non-steroidal anti-inflammatory drugs; Gastric bleeding; Gastric ulcer; EP4-subtype receptor

INTRODUCTION

Over 300 million patients use non-steroidal anti-inflammatory drugs (NSAIDs) in the world to treat pain, arthritis, fever and other diseases. Nearly 30% of the users suffer from gastric lesions and bleeding. Mechanisms for such actions of NSAIDs seem to be complex and multifactorial, including the inhibition of prostaglandin (PG) synthesis, induction of apoptosis and necrosis of gastric mucosal cells[5,7], neutrophil penetration, dysfunction of microvessels, reduced secretion of bicarbonate and mucus, and increased gastric motility[8].

Proton pump inhibitors (PPIs) have been the mainstay for the treatment of gastric ulcers, primarily due to its abilities to reduce acid secretion[9]. An alternative approach is to administer misoprostol, a non-selective prostaglandin E1 (PGE1) analogue. PGE2/E1 has been shown to protect isolated gastric glands from indomethacin, independently of neural, vascular and hormonal factors[10]. Misoprostol has successfully prevented NSAID-induced bleeding, perforation or gastric outlet obstruction in patients[6,7], and reversed the negative effects of indomethacin on the maturation of granulation...
with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. The cells were seeded in 6-well plates at 1 × 10^5 cells/well. After overnight (37 °C, 5% CO2) culture, the cells were treated with indomethacin at 50, 100, 200 or 400 μmol/L for 24 h, under serum-free conditions, to induce apoptosis as reported elsewhere[18]. Cell apoptosis was quantitated with flow cytometry as below. To evaluate EP4 agonist effect on cell survival, 70% confluent cells were treated with the EP4 agonist at 0, 1, 3 or 10 nmol/L, respectively, followed by 400 μmol/L indomethacin 30 min later. Twenty-four hours later, cells were collected and washed in cold PBS, and fixed with cold 70% ethanol added drop by drop while vortex stirring. Following overnight fixation, the cells were stained with propidium iodide (10 μg/mL, Sigma) and RNase A (1 mg/mL, Sigma) for 30 min in the dark. The cells were sorted by flow cytometry using CellQuest software (Becton Dickson, San Diego, CA). The sub-2N population was quantified. The percentage of apoptotic cells was calculated by sub-2N population from each drug treatment minus vehicle treatment[19].

Indomethacin-induced gastric damage model
High dose indomethacin (20 mg/kg in 4% DMSO, corn oil) was orally gavaged to induce gastric damage in C57BL/6 mice (Charles River, Wilmington, MA) at 8 wk old[20,21]. Vehicle or the EP4 agonist at 0.1 mg/kg in 0.1 mL 4% DMSO-corn oil was orally gavaged, 24 h and 30 min prior to indomethacin. Gastric lesions were assessed 24 h after indomethacin administration. The stomachs were then removed, inflated with 2% formalin, immersed in 2% formalin for 10 min and then opened along the great curvature. The area of hemorrhagic lesions was measured under a dissecting microscope (16 × magnification) with a square grid (×10), summed per stomach, and used as a lesion score[20,21]. The stomachs were then fixed in 10% formalin and sectioned at 5 μm thickness. HE staining was performed as usual. To monitor cell proliferation, BrdU was injected intraperitoneally at 10 mg/mL in 0.1 mL of normal saline, 16 h prior to sacrifice. Paraffin-embedded sections were deparaffinized in xylene and rehydrated in ethanol. Antigen was retrieved with citrate buffer, pH 6.0, boiled for 5 min in a microwave and slowly cooled down at room temperature. Immunofluorescence staining of BrdU was then performed following manufacturer’s instructions (Roche-Applied Science, Penzberg, Germany). Briefly, the sections were incubated with sufficient amount of PGE2 for FP, 17-phenyl PGF2α for FP, carbacyclin for FP and U-46619 for FP.

The EP4-selective agonist
Competition binding experiments were performed in a medium containing Hank’s balanced salt solution, 20 mmol/L HEPES, pH 7.3, membranes (about 60 μg protein) or 2 × 10^7 cells from HEK 293 cells stably expressing the human EP4 receptor, [3H] PGE2 (10 nmol/L) and various concentrations of test compounds in a total volume of 300 μL, read with LS6500 multi-purpose scintillation counter (Beckman Coulter, CA). cAMP assay was carried out using AlphaScreen cAMP assay kits (PerkinElmer, Boston, MA) following manufacturer’s instructions. Intracellular Ca^2+ was monitored using AlphaScreen cAMP assay kits (PerkinElmer, Boston, MA) following manufacturer’s instructions. Intracellular Ca^2+ was monitored using a FLIPR Tetra system and assay kits from Molecular Devices following manufacturer’s instructions. All assays were carried out in HEK-293 cells heterologously and stably expressing each of the eight human recombinant prostaglandin receptors. For Ca^2+ signals, hEP2, hEP4 and hDP were co-expressed with a chimeric G protein, Gq5, which converts the Gs signal to a Gq Ca^2+ signal, and hEP3 with a chimeric G protein, Gqi. Subtype-selective compounds used here were PGE2 for EP1, EP2, EP3 and EP4; BW245C for DP; 17-phenyl PGF2α for FP, carbacyclin for FP and U-46619 for FP.

The PGE2 analog used in this study bound hEP4 with a Ki of 6.7 ± 0.7 nmol/L, not other prostaglandin receptors, and increased cAMP production with an EC50 of 0.25 ± 0.03 nmol/L. On the other hand, the drug at 10 μmol/L showed no detectable FLIPR signals in HEK 293 cells heterologously expressing hEP1, hEP, hIP and hTP, and also in hEP2 (Gqs), hEP4 (Gqs), hEP3 (GQP), hDP (Gqs), respectively. This compound is unstable in liver microsomes and thus when locally applied, its systemic exposure was minimal.

Cell culture and apoptosis assay
Human gastric mucosal cells (AGS) were purchased from the American Type Culture Collection (Manassas, VA), and maintained on Ham’s F-12 medium (GIBCO-BRL)
sterilization with betadine and 70% ethanol, a midline incision was made to expose the stomach. Five microliters of 40% acetic acid was added through a 3 mm curette onto the serosal surface of the anterior wall of the stomach (just proximal to the antral gland area). The curette was placed tightly on the stomach surface to limit the spread of acetic acid. Thirty seconds later, acetic acid was wiped off and the surface was cleaned with normal saline. The abdomen wall was closed by 6-0 silk sutures, and the skin was closed by staples[21]. We first performed a study to monitor the dynamic changes of ulcers and animals. The mice lost some weight initially and recovered in 2 d. Their ulcer sizes were peaked at day 3 and then spontaneously healed within 2 wk. Vehicle or the EP4 agonist (0.1 mg/kg per day) and/or indomethacin (3 mg/kg per day) were orally gavaged from day 3 to day 6 in 0.1 mL 4% DMSO-corn oil. Then the animals were assessed on day 7. To study EP4 agonist effect on chronic gastric ulcer healing (without indomethacin), the EP4 agonist (0.1 mg/kg per day) or vehicle were given from day 3 to day 10, and evaluated on days 7 and 11, respectively.

On the day of sacrifice, blood was withdrawn, and hematologic analysis was conducted by personnel who did not know the treatments (ADVIA1A120 Hematology System, Bayer, Tarrytown, NY). The stomachs were inflated with 2% formalin for 10 min and opened along the greater curvature. The stomachs were flattened on 3M paper. The ulcers were photographed under dissection microscopy (× 16) with a hooked camera, and images stored in the computer and analyzed by SPOT software. The stomachs were then fixed in 10% formalin and processed for sectioning. A slice cutting through the biggest diameter of each ulcer was sectioned and stained by HE.

All animal use protocols were approved and performed according to the guidelines of Allergen's animal care and use committee. Data shown are mean ± SE. Statistical analysis was conducted by student t-test.

RESULTS

**EP4 agonist decreased indomethacin-induced apoptosis**
Exposure of human gastric mucous epithelial cells (AGS) to indomethacin (0, 50, 100, 200, 400 μmol/L) for 24 h concentration-dependently induced cell apoptosis as determined using flow cytometry analysis (Figure 1A). Particularly, indomethacin at 400 μmol/L markedly increased apoptosis, nearly 10-fold greater than in the untreated cells, and its activity was significantly reduced upon treatment (30 min before) with a highly-selective and potent EP4 agonist (see materials and methods), in a dose-dependent manner (Figure 1B). The EP4 agonist at the highest dose, 10 nmol/L, significantly decreased indomethacin-induced apoptosis, by more than 50%.

**EP4 agonist alleviated indomethacin-induced acute damage and promoted epithelial regeneration in mice**
In rats, apoptosis of mucous epithelial cells contributes to indomethacin-induced lesions in stomachs[18], and here we examined whether the EP4 agonist protects the gastric mucous layer from indomethacin. Indomethacin at high dose (20 mg/kg) produced band-shaped hemorrhagic lesions in the mucous layer mostly at the glandular part of the stomach, occurring 7 h post indomethacin application. Histologically, there was edema and disorganization of the mucous layer, patchy mucous epithelial cell exfoliation, shallow ulcer formation and bleeding with infiltration of inflammatory cells in vehicle treated mice. The mucous layer of EP4-treated mice remained largely intact except for some sparse, focal superficial defects in mucous cells (Figure 2A). Treatment with the EP4 agonist (concentration 0.002%), 24 h and 30 min before indomethacin, significantly reduced gastric lesion scores, from an average of 16 to less than 6 (Figure 2B). BrdU staining-positive cells were largely limited to the isthmus and neck region in the tubular glands of the stomach mucosa layer in vehicle-treated mice (Figure 2A). BrdU-positive cells migrated much higher and lower along the tubular glands in the EP4 agonist-treated mice than vehicle-treated mice (Figure 2A). BrdU-positive cells in mucous layer were on average 29% in EP4 treated mice, and 21% in vehicle treated mice (Figure 2B). Taken together, the EP4 agonist may stimulate proliferation and migration of gastric epithelial progenitors, so as to accelerate mucous repair.
Low dose indomethacin exacerbated chronic gastric ulcers in mice

A chronic gastric ulcer model was established by acetic acid application in mice. Low-dose indomethacin (3 mg/kg per day), which is sufficient to block de novo synthesis of PGE2, was applied 3 d post ulcer induction. On day 7, the indomethacin treatment increased gross ulcer areas by 76% as compared to vehicle-treated mice (P < 0.01, Figure 3A). Consistent with exacerbation of gastric ulcer sizes, hematology analysis revealed that indomethacin also worsened blood loss from the ulcers (Figure 3B), and higher lymphocyte surge as compared to untreated controls (Figure 3C). This supports the view that blocking of de novo synthesis of PGE2 delayed spontaneous repair of established gastric ulcer, and exacerbated inflammation and bleeding, which is similar to human gastric ulcer’s responses to NSAIDs.

EP4 agonist ameliorated indomethacin exacerbation on chronic gastric ulcer in mice

We next investigated whether exogenous EP4 agonist is capable of promoting ulcer healing in the presence of indomethacin treatment. Indomethacin (3 mg/kg) with EP4 agonist (0.002% in 0.1 mL) or with vehicle was orally administered to mice with established gastric ulcers from day 3 to day 7. Mice treated with EP4 agonist had a smaller ulcer size than mice treated with vehicle, 75.04% ± 7.06% and 100.02% ± 9.44%, respectively, on day 7. Hematology analysis showed that EP4 agonist treatment significantly ameliorated loss of red blood cells, hemoglobin and hematocrit (Figure 4A). This may suggest that EP4 agonist-treated mice had either smaller ulcers or more mature granulation tissue than control mice. EP4 agonist leads to gastric mucous vasodilation, not vasoconstriction (mediated by EP3 receptor)[13] and mature granulation tissue is more resistant to noxious stimuli. The inflammation at ulcer sites was reflected by white blood cell counts in the peripheral circulation. EP4 agonist treatment decreased white blood cell counts from 6900/μL to 5600/μL, and lymphocyte counts from 4690/μL to 3330/μL (P < 0.05) (Figure 4B).

EP4 agonist accelerated the spontaneous healing of chronic gastric ulcer

We also examined the effects of EP4 agonist alone on gastric ulcer healing. By day 7, treatment with EP4 agonist reduced ulcer area by 40% as compared to that observed with vehicle treated mice (Figure 5A, P < 0.005). By day 11, the drug further reduced ulcer size by 70% (Figure 5A). There was much less inflammatory cell infiltration and necrosis tissue in the ulcers of EP4 agonist-treated animals, compared with untreated mice.

Figure 2 EP4 agonist effect on indomethacin-induced gastric lesion in mice. EP4 agonist was orally administered 24 h and 30 min prior to indomethacin dosing at 20 mg/kg. The stomachs were assessed for mucus lesions 24 h after indomethacin dosing. A: HE and BrdU immunohistochemistry staining of stomachs (× 200). Superficial mucosal cells had sloughed off gastric mucus with infiltration of inflammatory cells in vehicle-treated group (arrow points to one lesion site). The mucus of EP4 agonist-treated stomachs was almost normal, except for a sparse focal defect of superficial mucous cells without inflammatory cells (arrow points to one lesion site). BrdU labeling showed robust mucous epithelial regeneration and migration in EP4 agonist-treated rats compared with that of vehicle treatment. B: Quantification of gross lesion under dissection microscopy (×16) and percentage of BrdU-positive cells among mucus cells. Shown are lesion scores, b P < 0.0001, n = 10; and BrdU percentage, d P < 0.01, n = 10, respectively.
On sectioning slides, inflammatory cell scores were 1.8 ± 0.2 for the EP4-agonist-treated and 2.7 ± 0.2 for the vehicle-treated mice (P < 0.05) (Figure 5B).

**DISCUSSION**

In the present study, we have shown that indomethacin, a prototypic cyclo-oxygenase (COX) inhibitor, at high dose, induced gastric epithelial apoptosis and produced gastric hemorrhagic lesions, and that the EP4-selective agonist we used here reduced such indomethacin-induced gastric injuries. Also, the EP4 agonist ameliorated ulcer bleeding and inflammation exacerbated by indomethacin at a low dose on existing ulcers, and promoted the spontaneous healing of chronic gastric ulcers in the absence of indomethacin. Such indomethacin-induced gastric injuries and PGE2 analogue-induced gastric protection appear to be somewhat similar to their actions observed at cellular level: NSAIDs are known to bring about mitochondrial damage, caspase cleavage, and eventually cell apoptosis in human gastric mucus as well as animal primary gastric epithelial cells. PGE2 and its analogs, on the other hand, inhibit indomethacin-induced mitochondrial damage and apoptosis in gastric epithelial cells, and a PGE1 analog, misoprostol, has been shown to reverse the inhibitory effect of NSAIDs on the regeneration of gastric mucous epithelial cells from human and animals. 11-deoxy-PGE1 (EP3/EP4 agonist) reverses indomethacin-induced delay in the healing of chronic gastric ulcers. One EP4-selective antagonist shows a deleterious effect on the spontaneous healing of chronic gastric ulcers with simultaneous suppression of vascular endothelial growth factor (VEGF) expression. Both pathways mediate pro-survival and proliferation signals in various epithelial cell lines, and are in line with our observation that the EP4 agonist inhibits indomethacin-induced apoptosis in human gastric mucosal cells (AGS), an established model for gastrointestinal effects of COX inhibitors, and expressing high levels of EP4.
transcripts (data not shown). Besides cell survival, the stimulation of intracellular cAMP from EP4 activation induces smooth muscle relaxation, and increases mucous blood supply and mucus secretion, which may additionally contribute to lessening indomethacin-induced injuries\(^{25,31-33}\).

In addition to acute damages produced by high-dose indomethacin, we also observed the chronic deleterious gastric effects of indomethacin at low-dose, which is more relevant to common clinical situations. Consistent with earlier reports\(^{17,34,35}\), low-dose indomethacin delayed healing of chronic gastric ulcers, exacerbated ulcer bleeding and inflammation, due to the inhibition of COX-2 expression and de novo synthesis of PGE2, and the inhibition of epithelial cell proliferation at the ulcer edge in both animals and humans. We have shown here that the EP4 agonist reversed such chronic effects induced by indomethacin at low dose.

Interestingly, we also observed here that the EP4 agonist accelerated the healing of chronic gastric ulcers under non-indomethacin challenged conditions. The major part of gastric ulcer healing is the restoration of gastric structure, which depends on the formation of the granulation tissue template made of gastric fibroblast cells and neovasculature. These fibroblasts express EP4 abundantly, and its activation is known to increase the synthesis of basic fibroblast growth factor, hepatocyte growth factor and VEGF\(^{29,36-38}\). All these factors may accelerate regeneration of fibroblasts and extracellular matrix, thus restoring ulcerated areas, and restituting epithelial cell layers\(^{17,38}\). Also the EP4 agonist showed anti-inflammatory activities as shown here and reported earlier\(^ {19}\): fewer inflammatory cells in the blood and minimal infiltration in the ulcers from animals treated with the EP4 agonist. This should facilitate the healing process since the control of inflammation is a pre-requisite to rapid healing\(^ {35}\).

In summary, EP4 agonists may mimic the gastric protective effects of PGE2 in the presence or absence of NSAIDs, and may show advantages over non-selective analogs such as misoprostol by minimizing adverse effects arising from activating all 4 subtype receptors of PGE2.

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COMMENTS

Background
Non-steroidal anti-inflammatory drugs (NSAIDs) including both cyclooxygenase (COX)-1/2 and COX-2-selective inhibitors, such as indomethacin, ibuprofen and celecoxib, have been prescribed in the world for treatment of pain, arthritis, menstrual symptoms and cancer, to name a few. However, nearly 30% of patients suffer from gastric lesions and bleeding. To mitigate NSAIDs’ adverse effects on the stomach, misoprostol, a non-selective PGE1 analogue, has been prescribed as the first choice for prevention of NSAID-induced injuries in USA, but often induces severe adverse effects. There remain unmet medical needs for drugs with improved therapeutic profiles.

Research frontiers
PGE2/E1 interacts with 4 subtype receptors, EP1, 2, 3 and 4 in mammalian cells. Numerous studies have been performed to understand each subtype receptor’s function and mechanism under various physiological and pathological conditions. High subtype receptor-selective ligands have been designed and tested to avoid adverse effects from non-selective drugs, such as misoprostol.

Innovations and breakthroughs
This study employed a novel, highly selective EP4 agonist, reported direct evidence for its protective activities against NSAIDs in the stomach, and further disclosed that the EP4 agonist may in part function through promoting mucous
epithelial cell survival and regeneration. This is the first study to show that an EP4 agonist may facilitate chronic gastric ulcer healing, although similar activity of EP4 in the stomach has been implicated in several concurrent studies using non-selective EP4 agonists or antagonists.

**Applications**

The concept from this paper would facilitate therapeutic developments of EP4-selective agonists for prevention of NSAIDs’ adverse effects in the gastrointestinal (GI) tract as well as for monotherapy treatment of gastric ulcers. Further, EP4 agonists may provide gastric protection under conditions such as stress, radio/chemotherapy and other conditions compromising GI activities.

**Terminology**

Prostaglandin E2 (PGE2) is synthesized via key enzymes COX-1/2, under normal conditions primarily by COX-1 and under pathological conditions by inducible COX-2. PGE2 is a paracrine or autocrine hormone, and is involved in inflammation and pain, and also plays an important role in functional stability of the GI tract.

**Peer review**

In this manuscript, the authors investigated the effects of EP4-selective agonist on indomethacin-induced gastric lesions and spontaneous healing of chronic gastric ulcers in mice or cultured human gastric mucous cells. The study was well performed and very interesting.

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