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The protective effect of rebamipide on paracellular permeability of rat gastric epithelial cells

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SUMMARY

Background: Barrier function in gastric epithelial cells is essential for the gastric defence mechanism against acid back-diffusion into the mucosal layer. Our previous study indicated that trans-epithelial resistance (TER) of rat gastric epithelial cells was rapidly increased when the cells were exposed to acid. This response to acid was diminished by indometacin.

Aim: Evaluate the effects of a mucoprotective agent, rebamipide, on the nonsteroidal anti-inflammatory drug (NSAID)-induced increase of gastric epithelial permeability.

Methods: Rat gastric epithelial cells were plated on tissue culture inserts. Cells were exposed to a NSAID (indometacin, 10^{-7} M). Trans-epithelial permeability was measured by TER and diffusion rate of ^{14}C-mannitol. The effect of rebamipide was evaluated by measuring TER. Endogenous prostaglandin E_2 (PGE_2) production in culture medium was also measured.

Results: Indometacin gradually and significantly decreased TER and increased ^{14}C-manitol permeability. Rebamipide reversed the indometacin-induced changes in epithelial permeability and induced PGE_2 synthesis. This induction was blocked by either indometacin or a Cyclooxygenase (COX)-2 specific inhibitor.

Conclusions: COX inhibitors such as indometacin inhibit regulation of epithelial permeability by reducing PGE_2. COX-1 has an important role in the gastric defense mechanism. Rebamipide suppressed an indometacin-induced increase in gastric epithelial permeability by increasing PGE_2 levels in a COX-2 dependent manner.

INTRODUCTION

Prostaglandins such as Prostaglandin E_2 (PGE_2), applied exogenously, have exhibited highly active role in preventing gastric mucosal damage.[1–4] Endogenous prostaglandin synthesis also has an important role in gastric mucosal defense.[5, 6] Prostaglandins are synthesized by cyclooxygenase (COX) which has two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most cells, whereas COX-2 is induced in gastric epithelial cells by various stimuli *in vitro*[7, 8] as well as *in vivo*.[9] COX-2-specific inhibitors delay healing of gastric injury in experimental animals,[9, 10] suggesting an important role for this isozyme in gastric ulcer healing.

Rebamipide, 2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinone-4-yl] propionic acid, is a gastroprotective agent used in Japan for the treatment of gastritis and gastric ulcers. Rebamipide has been reported to provide gastroprotection through scavenging of free-radicals[11–13] and has also prevented various experimental delayed wound repair *in vitro*[14–16] as well as *in vivo*.[17–24] This compound also increases prostaglandin levels in gastric tissue and protects gastric mucosa from various injuries.[19] Despite the accumulation of a substantial body of data regarding protective action of rebamipide, the

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underlying mechanisms through which it exerts protective effects in the injured stomach are not well understood.

In this study, we examined how epithelial permeability was affected when gastric epithelial cells were exposed to nonsteroidal anti-inflammatory drugs (NSAIDs) with or without rebamipide, and the role of prostaglandins on these cell permeability responses.

MATERIALS AND METHODS

Rat gastric epithelial cell line (RGM-1)

The rat gastric epithelial cell line (RGM-1; Riken Cell Bank, Tsukuba, Japan; provided by Dr Matsui)[25] was used in this study. RGM-1 was cultured in Dulbecco's modified eagle medium (DMEM; Gibco BRL, Grand Island, NY, USA) and F-12 nutrient mixture (Gibco BRL) (1 : 1), supplemented with 20% fetal bovine serum (FBS; Bio Whittaker, Walkersville, MD, USA) and antibiotic-antimycotic (Gibco BRL) at 37 °C in 5% CO_2 incubator.

Trans-epithelial Electrical Resistance (TER)

RGM-1 were plated on to 9 mm diameter, 8 μm pore size tissue culture inserts (Becton Dickinson, Franklin Lakes, NJ, USA) coated with fibronectin (5 μg/mL). The inserts were placed in 24-well plates containing 1 mL of culture medium, and then the cells were incubated for 3 or 4 days at 37 °C in a 5% CO_2 incubator. At 100% confluence, TER was measured using the MILLICELL-ERS (Millipore Corporation, Bedford, MA, USA) with a lab–modified barrier device in which 'WPI chopstick' electrodes STX-02 (WPI, New Heaven, CT, USA) were fixed at a set distance 2 mm from the apical and basal sides of the monolayer. The values obtained from a blank insert coated with fibronectin were subtracted to give the net resistance, which was multiplied by the membrane area to give the resistance in are-corrected units. Values were expressed as $\Omega \cdot cm^2$, taking into account the surface area of the filter (0.38 cm^2). We found that there was some basal variation in the resistance of each filter alone. Therefore we normalized the initial baseline resistances for each insert, to account for these slightly different baseline resistance values. The change of electrical resistance was represented by percentage baseline resistance.

The percentage of baseline resistance was calculated as follows:

$$\begin{aligned}\%\ \text{baseline resistance} = &[\text{resistance from each time point}] \\ &- [\text{resistance from a blank insert coated with fibronectin}]/ \\ &[\text{baseline resistance}] \\ &- [\text{resistance from a blank insert coated with fibronectin}] \\ &\times 100\end{aligned}$$

where baseline resistance was the resistance from 0 time point.[26]

When resistance was stable (at $> 12\ \Omega \cdot cm^2$), the culture medium from the upper compartment of the monolayer was removed and replaced with fresh medium. In some experiments, monolayers were treated with rebamipide, 2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinone-4-yl] propionic acid (obtained from Otsuka Pharmaceutical Co., Ltd, Toskushima, Japan), with or without indometacin (10^{-7} M) (Wako Co., Ltd, Tokyo, Japan) or NS-398 (10^{-7} M) (a specific COX-2 inhibitor; Calbiochem., Darmstadt, Germany). In a series of experiments PGE_2 (10^{-6} M) (Wako Co., Ltd, Tokyo, Japan) was added every 15 min in the presence of indometacin. All data represent the average of four identically treated monolayers.

Trans-epithelial permeability

Epithelial monolayers grown on the surface of membrane filters were washed once with medium, and then placed in 24 well plates (Becton Dickinson) filled with 1 mL of medium per well. Medium (500 μL) containing 9250 Bq/mL ^{14}C-Mannitol was placed in the top chamber and the apparatus was placed in a CO_2 incubator. After incubation, every 15 min, 10 μL samples from both chambers were transferred to other tubes. Then, the amount of ^{14}C-Mannitol that passed across an epithelial monolayer, as well as the amount that did not pass, were measured. The diffusion rate of ^{14}C-Mannitol was expressed as follows:

$$\begin{aligned}&\text{Permeability index (\%)} \\ &= [^{14}\text{C-Mannitol in the lower chamber}]/ \\ &\quad [^{14}\text{C-Mannitol in the lower chamber}] \\ &\quad + [^{14}\text{C-Mannitol in the upper chamber}]\end{aligned}$$

PGE_2 production

To measure endogenous PGE_2 production in the medium of RGM-1, the culture medium was removed and replaced with fresh medium 1 h before the

treatment. At time 0, cells were treated with rebamipide (1 mM) with or without indometacin or NS-398. After 1 h of incubation, media were collected and PGE_2 level in the medium was measured using enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI, USA).

Statistical analysis

All values are expressed as means ± standard error (S.E.). Unless otherwise stated mean values were compared by one-way ANOVA with Bonferroni's correction for multiple comparisons. In TER experiments, values for each monolayer were normalized to the initial baseline value, which was set as '100%'. Changes in monolayer resistance were calculated as the percent change relative to controls and were analysed by one-way ANOVA with Bonferroni's correction. Probability (P) values of < 0.05 were considered significant.

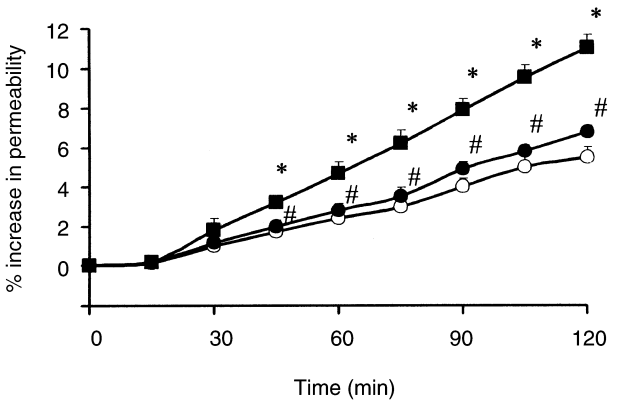

Figure 2. Effect of rebamipide on ^{14}C-mannitol permeability. ^{14}C-manitol was used as a tracer. In control conditions (○), ^{14}C-mannitol permeability gradually increased over time. Indometacin (10^{-7} M) (■) induced a significant increase in ^{14}C-mannitol permeability (from 45 min after stimulation up to 120 min) compared with controls ($P < 0.05$). Treatment with rebamipide (1 mM) (●) blocked indometacin-induced increase in ^{14}C-mannitol permeability.

RESULTS

Effects of rebamipide on indometacin-induced TER decrease and permeability increase

When the cells were treated with a nonspecific inhibitor against COX-1 and -2, indometacin (10^{-7} M), the TER of RGM-1 was significantly decreased compared with controls (Figure 1). The decrease of TER induced by indometacin, however, was significantly blocked by rebamipide (1 mM) (Figure 1). In a preliminary study 1 mM rebamipide has a maximal effect on gastric epithelial cells *in vitro* and this concentration was not toxic for the cells (data not shown). ^{14}C-mannitol was also used as a tracer for measuring paracellular permeability of RGM-1. In control conditions (pH 7.4), ^{14}C-mannitol permeability gradually increased over time. We performed the ^{14}C-mannitol permeability assay with indometacin and rebamipide (Figure 2). Indometacin (10^{-7} M) induced a significant increase in ^{14}C-mannitol permeability (from 45 min after stimulation up to 120 min) compared with controls ($P < 0.05$). Pre-treatment with rebamipide (1 mM) significantly blocked the indometacin-induced increase in ^{14}C-mannitol permeability (Figure 2). These data are consistent with data from the TER experiment (Figure 1).

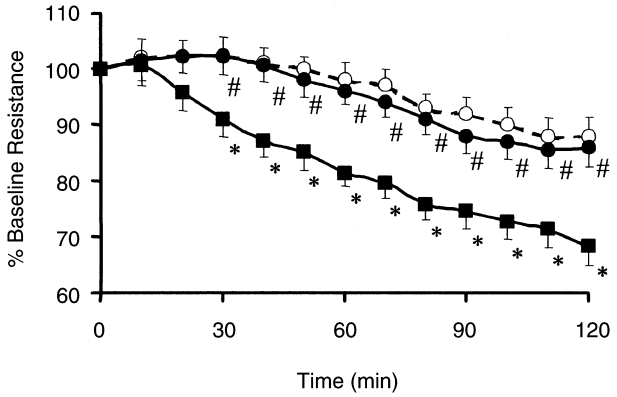

Figure 1. Effect of rebamipide on indometacin-induced TER decrease. Indometacin (10^{-7} M) (■) induced a significant decrease in TER (from 30 min after stimulation up to 120 min). Treatment with rebamipide (1 mM) (●) blocked indometacin (10^{-7} M)-induced decrease of TER. Data were obtained from four separate experiments and expressed as mean ± S.E. ○: control. $^{*}P < 0.05$ vs. control (○). $^{\#}P < 0.05$ vs. indometacin (10^{-7} M).

Effects of rebamipide, indometacin and NS-398 on PGE_2 levels in RGM-1

PGE_2 was not present in the fresh RGM-1 medium. When the cells were incubated with fresh medium for 60 min, the concentration of PGE_2 in the medium was 107 ± 7.6 pg/mL (control) (Figure 3). In control condition, RGM-1 continuously produces PGE_2. Treatment of indometacin (10^{-7} M) significantly reduced PGE_2 production of RGM-1 (75 ± 7.8 pg/mL) ($P < 0.05$). On

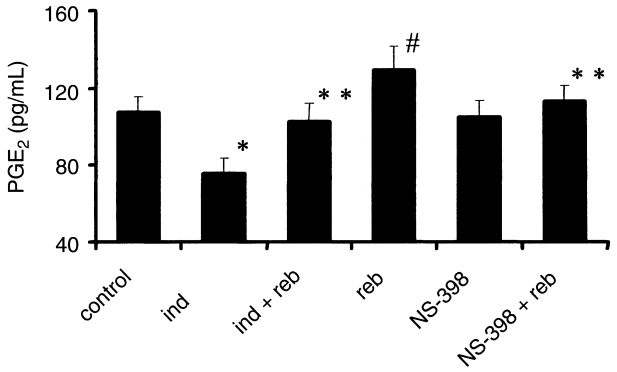

Figure 3. Effects of rebamipide, indometacin and NS-398 on PGE_2 levels in RGM-1. When the cells are incubated with fresh medium for 60 min, the concentration of PGE2 in the medium was 107 ± 7.6 pg/mL (control). Treatment of indometacin (ind) significantly reduced PGE_2 production of RGM-1 cells (75 ± 7.8 pg/mL) ($P < 0.05$). On the other hand, a specific COX-2 inhibitor, NS-398, did not alter PGE_2 production (113 ± 7.6 pg/mL). PGE_2 levels in medium were significantly higher in rebamipide (reb) treated condition (130 ± 11.7 pg/mL) than that in control condition ($P < 0.05$). Indometacin blocked rebamipide-induced PGE_2 production to the control level ($P < 0.05$). NS-398 significantly reduced rebamipide-induced PGE_2 production ($P < 0.05$). $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. control. $^{**}P < 0.05$ vs. rebamipide (reb) (10^{-7} M).

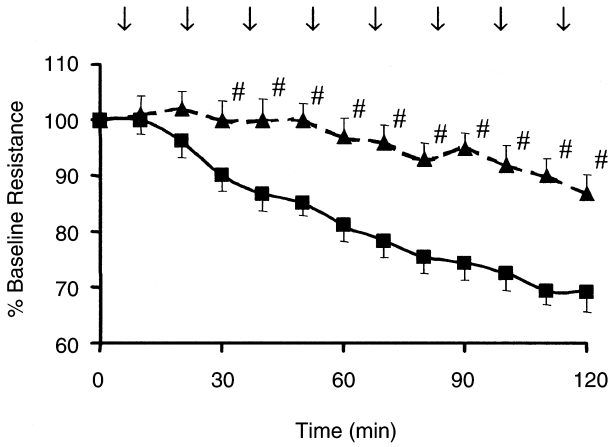

Figure 4. Effect of PGE_2 on indometacin-induced TER. Indometacin (10^{-7} M) (■) induced gradual decrease in TER (from 30 min after stimulation up to 120 min). PGE_2 was added to the well every 30 min for 120 min in the presence of indometacin (10^{-7} M). Treatment of PGE_2 (10^{-6} M) every 15 min (↓) in the presence of indometacin (10^{-7} M) reversed the indometacin-induced decrease of TER (▲). Data were obtained from four separate experiments, and expressed as mean ± S.E. $^{\#}P < 0.05$ vs. indometacin (10^{-7} M).

the other hand, a specific COX-2 inhibitor, NS-398 (10^{-7} M), did not alter the PGE_2 production (113 ± 7.6 pg/mL). These data indicate that COX-1 has an important role in the PGE_2 production in the control condition. PGE_2 levels in medium were significantly higher in the rebamipide (1 mM) treated condition (130 ± 11.7 pg/mL) than in the control condition ($P < 0.05$). Indometacin blocked rebamipide-induced PGE_2 production to control level (103 ± 9.3 pg/mL) ($P < 0.05$). NS-398 significantly reduced rebamipide-induced PGE_2 production (113 ± 7.5 pg/mL) ($P < 0.05$). These data indicate that COX-2 is connected to rebamipide-induced PGE_2 production.

Effects of PGE_2 on indometacin-induced TER decrease

To investigate the effects of PGE_2 on gastric epithelial cells, PGE_2 was added every 15 min. When the cells were treated with PGE_2 (10^{-6} M) every 15 min for 120 min in the presence of indometacin (10^{-7} M), the indometacin-induced decrease of TER was abolished ($P < 0.05$) (Figure 4). PGE_2 itself had no direct effect on TER under control conditions (data not shown).

DISCUSSION

In recent years, the understanding of pathophysiology and treatment of peptic ulcer diseases has changed remarkably. It is now clear that the major cause for gastric and duodenal ulcers is the infection of *Helicobacter pylori*. Effective eradication therapies against *H. pylori* have been developed, and with these therapies most problems concerning gastric and duodenal ulcers seem to be disappearing. One exception, however, is the ulcers caused by the use of NSAIDs. To prevent NSAID induced gastric injury, acid suppressive drugs such as H_2-receptor antagonist and proton pump inhibitor are usually employed. Recent reports demonstrate that a mucoprotective agent, rebamipide, also protects gastric mucosa against use of NSAIDs in humans.[27] We therefore examined the effect of rebamipide on changes in paracellular junctional permeability when gastric epithelial cells are exposed to NSAIDs.

An enzyme responsible for the synthesis of physiologically important prostaglandins, COX, is well known to play a key role in maintaining the integrity of gastric mucosa.[28] An isoform, COX-1, is constitutionally expressed in most cell-types to produce prostaglandins which are essential for cell homeostasis, whereas COX-2 expression is induced by growth factors, cytokines, and lipopolysaccharides.[29] COX-2 is considered to play an

important role in gastric ulcer healing since COX-2-specific inhibitors delay healing of gastric injury in experimental animals.[9, 10] RGM-1 cells were known to have cell polarity as gastric epithelial cells and prostaglandin receptors. They also secrete mucus in response to stimulation by prostaglandins.[30] It has also been noted that RGM-1 cells express COX-1 at a high level in their normal state and can also induce expression of COX-2 when stimulated appropriately.[31–33]

Our present results demonstrate that NSAIDs like indometacin inhibit COX-1 dependent monolayer integrity (seen as an decrease in TER) and also increase paracellular permeability (Figure 1). Alone, NS-398, a COX-2-specific inhibitor did not significantly alter the TER (data not shown). The difference between the effects of indometacin and NS-398 might be explained by differences in COX-1 and COX-2 expression in these cells, which controls the production of prostaglandins.

Previous studies have shown that PGE_2 stimulates increased intracellular Ca^{2+} and cAMP and also promotes sealing of tight junctions.[34, 35] These data indicate that the abnormal paracellular permeability caused by NSAIDs is not a direct cell injury action by NSAIDs, but a failure of the cells to produce prostaglandins (especially PGE_2 due to inhibition of the COX activity). The concentrations of NSAIDs used in previous studies were 10^{-4}–10^{-5}.[36] Since the concentration of NSAIDs used in this experiment was very low (10^{-7} M), it is considered that a similar abnormality in gastric epithelial permeability is induced in humans taking NSAIDs. Rebamipide could reverse the PGE_2 production, which was partially blocked by a low dose of indometacin (10^{-7} M) (Figure 3). In addition, our data also showed that alternations in normal gastric TER caused by NSAIDs could be reversed by supplementing with PGE_2 (Figure 4). These data indicate that rebamipide protects gastric mucosa against NSAIDs (around 10^{-7} M) by keeping PGE_2 Levels.

We conclude that: (1) administration of NSAIDs that inhibit COX-1 significantly lowered gastric mucosal cells' junctional solute barrier; and that (2) rebamipide protects the gastric epithelial barrier against NSAID-induced injury by enhancing the junctional solute barrier through keeping of PGE_2.

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