Gastric mucosal protection against ethanol by EP$_2$ and EP$_4$ signaling through the inhibition of leukotriene C$_4$ production

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EP receptors are classified into four subtypes, EP$_1$, EP$_2$, EP$_3$, and EP$_4$. We have tested which EP receptor subtypes participate in gastric mucosal protection against ethanol-induced gastric mucosal injury and clarified the mechanisms of such protection. The gastric mucosa of anesthetized rats was perfused at 2 ml/min with physiological saline, agonists for EP$_1$, EP$_2$, EP$_3$, and EP$_4$, or 50% ethanol, using a constant-rate pump connected to a cannula placed in the esophagus. The gastric microcirculation of the mucosal base of anesthetized rats was observed by transillumination through a window made by removal of the adventitia and muscularis externa. PGE$_2$ and subtype-specific EP agonists were applied to the muscularis mucosae at the window. Application of 50% ethanol dilated the mucosal arterioles and constricted the collecting venules. Collecting venule constriction by ethanol was completely inhibited by PGE$_2$, and by EP$_3$ and EP$_4$ agonists (100 nM) but not by an EP$_1$ or an EP$_3$ agonist. Ethanol-induced mucosal injury was also inhibited by EP$_2$ and EP$_4$ agonists. When leukotriene (LT)C$_4$ levels in the perfusate of the gastric mucosa were determined by ELISA, intragastric ethanol administration elevated the LT$_C$4 levels sixfold from the basal levels. These elevated levels were significantly (60%) reduced by both EP$_2$ and EP$_4$ agonists but not by other EP agonists. Since LT$_C$4 application at the window constricted collecting venules strongly, and an LTC$_4$ antagonist reversed the LT$_C$4-induced mucosal injury, reductions in LT$_C$4 generation in response to EP$_2$ and EP$_4$ receptor signaling may be relevant to the maintenance of gastric mucosal microcirculatory integrity, which is a major determinant of protective action. We previously reported that gastric mucosal exposure to ethanol caused mucosal damage as a result of congestion of the gastric mucosal microcirculation. Congestion of mucosal blood flow, if persistent, induced intense hypoxia of the gastric mucosa, and as a result, the epithelium of the mucosa was damaged (32, 33). A prostaglandin I$_2$ (PGI$_2$) analog, beraprost sodium, inhibited ethanol-induced gastric mucosal injury through the increased release of a neuropeptide, calcitonin gene-related peptide (CGRP) (39). This was due to the action of CGRP in blocking ethanol-induced constriction of collecting venules and other venules. PGE$_2$ exhibited the same protective action against ethanol, but this PGE$_2$-dependent protection was not mediated by CGRP, since administration of a CGRP antagonist, CGRP-(8–37) did not block PGE$_2$-dependent protection. These observations indicated that PGE$_2$ exhibited protective action independent of CGRP. PGs exert their biological actions by binding to specific receptors that contain seven transmembrane domains. PGE$_2$ receptors have been defined pharmacologically and cloned, including four subtypes of PGE receptor (EP$_1$, EP$_2$, EP$_3$, and EP$_4$) (30). Genes for each of these receptors have been disrupted, and the corresponding knockout mice have been produced (1, 19, 22, 25, 26, 28, 40, 45). Furthermore, with the use of the cloned receptors, agonists and antagonists highly selective for each of the four EP subtypes have been or are in the process of being developed (41, 47). It is unlikely that any previous pharmacologic tools would neatly prevent clinical gastric mucosal lesions due to ethanol. Thus some effective drugs will be expected. In the present study, we have now identified the EP receptors responsible for the protection of gastric microcirculatory dysfunction with the use of selective compounds for EP receptor subtypes. Responsible EP agonists will be promising agents to prevent ethanol-induced gastric mucosal injury. Our results further clarified that the mechanisms of the protective actions of EP receptor signaling against the ethanol were leukotriene (LT)$C_4$ dependent and suggest that the agents acting on EP$_2$ and EP$_4$ will be therapeutically active against gastric microcirculatory dysfunction.

G80

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MATERIALS AND METHODS

Intravital Microscopy

Observation of rat gastric microcirculation. Male Sprague-Dawley rats (specific pathogen free, 250–450 g) obtained from Japan SLC (Hamamatsu, Japan) were housed at constant room temperature (25 ± 1°C) and humidity (60 ± 5%) with a 12-h light-dark cycle. Rats were deprived of food 18–24 h before the start of experimentation but had free access to water. The mucosal microcirculation of rats anesthetized with urethane (Aldrich, Milwaukee, WI) (0.875 g/kg ip) was observed through a modification improving on the method originally reported by Rosenberg and Guth (14, 23, 32, 33, 38).

Surgical preparation for gastric microcirculation experiments. After laparotomy with an electric cautery scalpel (model B-365; Takahashi Shoten, Tokyo, Japan), the greater curvature of the stomach was incised longitudinally leaving the greater omentum attached to the posterior wall while the anterior wall was resected. The dorsal side of the glandular stomach was secured in a plastic perfusion chamber, with the mucosal side facing the interior of the chamber, and was perfused with Tyrode’s solution at 37°C (Fig. 1). A small part of the serosa, together with the underlying muscularis externa layers and the submucosa, was carefully dissected away using microsurgical scissors under a stereomicroscope to make an observation window (Fig. 1).

This method made possible direct observation, with transillumination under a stereomicroscope to make an observation window. Ethanol solution under a stereomicroscope, as described in MATERIALS AND METHODS. A small part of the serosa, together with the underlying muscularis externa layers and the submucosa, was carefully dissected away using microsurgical scissors under a stereomicroscope to make an observation window. Ethanol solution was given to the mucosa, and prostaglandin E (PGE2) and EP agonists were administered at the window. EtOH, ethanol.

Fig. 1. Schema for the experimental setup to observe the microcirculatory dysfunction elicited by ethanol. A small part of the serosa, together with the underlying muscularis externa layers and the submucosa, was dissected away under the stereomicroscope, as described in MATERIALS AND METHODS. A small part of the serosa, together with the underlying muscularis externa layers and the submucosa, was carefully dissected away using microsurgical scissors under a stereomicroscope to make an observation window. Ethanol solution was given to the mucosa, and prostaglandin E (PGE2) and EP agonists were administered at the window. EtOH, ethanol.
of the perfusate were collected repeatedly at 2-min intervals and were placed directly into a plastic tube kept on ice.

**Measurement of intragastric LTC$_4$ levels.** The levels of LTC$_4$ in the perfusate from anesthetized rats were measured as described by us previously. Briefly, the perfusate for every 2 min was collected directly in ice-cold absolute ethanol, and after overnight centrifugation at 3,000 g at 4°C, the supernatant was evaporated at reduced pressure. The residue was applied to a Sep-Pack C18, and the resulting fractions for LTC$_4$ were determined by enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI 48108).

**Assessment of gross lesions in the glandular stomach.** At the end of each perfusion experiment, the stomach was excised, and the reddened areas were calculated as percentages of the glandular stomach area using Adobe Photo Shop 4.0f software on a Macintosh computer.

**Effects of an LT antagonist on ethanol-induced gastric mucosal injury.** An LT antagonist (ONO-1078, 0.03–0.3 mg/kg ip) was given to rats 1 h before ethanol administration, and the area of 50% ethanol-induced gastric mucosal injury was determined as described above.

**Immunohistochemistry of 5-Lipooxygenase**

The stomachs were immediately fixed with 4% paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.4). After fixation, the stomach tissues were dehydrated with a graded series of ethanol solutions, and then embedded in paraffin. The sections (4 μm) from the paraffin-embedded tissues were mounted on glass slides, deparaffinized with xylene, and then placed in cold (4°C) acetone for immunostaining.

The procedure for staining dehydrated sections using a Vectastain ABC Kit (Vector Laboratory, Burlingame, CA) was as follows: 1) incubation with diluted normal horse serum, 2) incubation with diluted (X500) 5-lipooxygenase polyclonal antibody (a gift from Prof. Ueda, Kagawa University, Kagawa, Japan), 3) incubation with biotinylated anti-IgG, 4) incubation with avidin-biotin-peroxidase complex, 5) placement in 0.02% 3,3’-diaminobenzine (DAB) and 0.3% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.4), 6) color development by immersion in DAB solution containing 0.005% H$_2$O$_2$, and 7) examination and photomicrography with a light microscope.

**Statistics**

All values in the figures are expressed as means ± SE of n observations. Statistical comparisons for multiple groups were made using one-way ANOVA with a posthoc Scheffe’s test. Values of P < 0.05 were considered statistically significant.

**RESULTS**

**Effects of PGE$_2$ administered at the window on gastric mucosal microcirculation.** Figure 2 summarizes the changes in diameter of arterioles 4 min after the administration of PGE$_2$. Application of PGE$_2$ induces concentration-dependent dilatation of arterioles in the basal part of the mucosal microcirculation. PGE$_2$ at concentrations of more than 1 μM/l dilated the arterioles significantly. By contrast, collecting venules and other venules were not dilated but were a little constricted at high concentrations.

**Effects of EP-selective agonists administered at the window on gastric mucosal microcirculation.** We examined changes of the diameters of microvessels in the basal part of the mucosal microcirculation when each EP receptor agonist alone was applied to the muscularis mucosae at the window. When EP$_1$ agonist or EP$_3$ agonist was added, the arterioles were not dilated, but EP$_2$ agonist dilated them significantly. EP$_4$ agonist at a high dose (1 mM) dilated arterioles (Fig. 3A). In the collecting venules, no agonist affected the diameters of the microvessels significantly (Fig. 3B). However, EP$_3$ agonist caused slight but significant constriction at a high concentration (100 μM). In the venules, there were few changes in the diameters of microvessels with the application of each EP agonist (Fig. 3C).

**Effect of PGE$_2$ applied at the window on the changes elicited by 50% ethanol in the diameter of gastric microvessels.** We examined the actions of PGE$_2$ (100 nM) on the changes elicited during mucosal application of 50% ethanol in the diameter of microvessels. When PGE$_2$ was administered at the window before mucosal exposure to 50% ethanol, ethanol-induced rapid constrictions of the collecting venules and venules were completely inhibited (Figs. 4, B and C). On the other hand, the diameters of arterioles during ethanol exposure were not affected by preadministration of PGE$_2$ (Fig. 4A, see data before ethanol administration). A concentration of 100 nM did not dilate arterioles under normal conditions. Further, even when we applied PGE$_2$ to the muscularis mucosae at the window during ethanol administration, it did not further dilate the arterioles (Fig. 4A).

**Effects of selective EP agonists administered at the window on changes in diameter of gastric microvessels elicited by 50% ethanol.** Further, we tested specification of EP receptor subtypes relevant to gastric mucosal protection to show inhibition of constriction of collecting and other venules (Figs. 5, B and C). We selected a concentration of 100 nM, which did not dilate the arterioles in the normal gastric microcirculation (Fig. 3A), since our preliminary experiments using different concentrations of PGE$_2$ or EP agonists (EP$_2$ and EP$_4$) described below indicated that 100 nM was enough to show the effects. The dilatation of arterioles elicited by 50% ethanol exposure increased the original diameter by about 100%, and this was not affected by prior administration of any EP selective agonist (Fig. 5A). On the other hand, the intense constrictions of the collecting venules and venules were completely blocked by either EP$_2$ or EP$_4$ agonists (Fig. 5, B and C). Administration of an EP$_1$ agonist or an EP$_3$ agonist...
Effects of PGE2, EP1, EP2, EP3, and EP4 agonists on 50% ethanol-induced mucosal injury. In rats, exposure of the gastric mucosa to 50% ethanol resulted in gastric mucosal lesions with a total reddened area covering 22.0 ± 0.645% (n = 6) of the area of the glandular stomach (Fig. 6). Prior intragastric administration of PGE2 (100 nM) significantly reduced the size of the mucosal lesions (7.83 ± 0.743%; n = 6) induced by mucosal exposure of 50% ethanol. Prior administration of EP2 agonist (7.77 ± 0.909%; n = 6) or EP4 agonist (7.62 ± 0.721%; n = 6) inhibited gastric mucosal lesions to the same degree as PGE2 (Fig. 6). However, the administration of EP1 agonist (22.0 ± 1.65%; n = 6) or EP3 agonist (21.0 ± 1.805%; n = 6) did not reduce the mucosal injury (Fig. 6).

Effects of PGE2 and EP agonists on the intragastric levels of LTC4. Intragastric LTC4 levels during perfusion of physiological saline were kept fairly constant; however, the exposure of the gastric mucosa to 50% ethanol after the perfusion of physiological saline immediately increased these levels by six times from the basal levels (Fig. 7A). As Fig. 6B shows, preadministration of PGE2 markedly inhibited the increase in LTC4 levels elicited by 50% ethanol. Similarly, the intragastric pre-exposure to an EP2 agonist and an EP4 agonist reduced the release of LTC4 levels elicited by 50% ethanol. By contrast, an EP1 agonist and an EP3 agonist did not significantly inhibit the release of LTC4.

Effects of LTC4 on gastric microcirculation. To evaluate the roles of LTC4 released by 50% ethanol, we tested first the effects of LTC4 applied to the muscularis mucosae at the...
window on the gastric microcirculation. As shown in Fig. 8, LTC₄ constricted collecting venules and venules in a concentration-dependent manner. These changes in gastric microcirculation were abolished with the simultaneous administration of LTC antagonist, ONO1078.

**DISCUSSION**

Disturbances in the gastric mucosal microcirculation are thought to be an important cause of injury, and observation of the microcirculation is therefore important for revealing the pathophysiology of mucosal injury (32, 33). As described above, 50% ethanol applied to the gastric mucosa induces mucosal congestion as a result of constriction of the collecting venules and other venules. We have previously reported that capsaicin applied to the mucosa prevented gastric lesions (2, 8). The importance of the cancellation of constriction of the collecting and other venules by capsaicin was confirmed by the action of CGRP, a major neuropeptide released by capsaicin. PGE₂, when administered exogenously, inhibits gastric mucosal injury, but this action was not inhibited by a CGRP antagonist, although the inhibition due to a PGI₂ analog was inhibited by CGRP antagonist (2, 8).

**Fig. 5.** Effect of selective EP agonists on ethanol-induced changes in the diameters of arterioles (A), collecting venules (B), and venules (C). Selective EP agonists were administered at the window prior to the mucosal application of 50% ethanol. Constriction of the collecting venules and venules were inhibited by an EP₂ agonist and an EP₄ agonist. The values are expressed as means ± SE from 5 rats. ANOVA was used for statistical analysis. *P < 0.05, **P < 0.01; vs. 50% EtOH alone.

**Fig. 6.** Effects of intragastric perfusion of PGE₂ and selective EP agonists on gastric lesions induced by 50% ethanol. The area of reddened lesions after 50% ethanol perfusion was determined during continuous infusion of physiological saline, PGE₂, or selective EP agonists. The values are expressed as means ± SE from 6 rats. ANOVA was used for statistical analysis. *P < 0.05; vs. 50% EtOH alone. NS, not significant.

**Fig. 5.** Effect of selective EP agonists on ethanol-induced changes in the diameters of arterioles (A), collecting venules (B), and venules (C). Selective EP agonists were administered at the window prior to the mucosal application of 50% ethanol. Constriction of the collecting venules and venules were inhibited by an EP₂ agonist and an EP₄ agonist. The values are expressed as means ± SE from 5 rats. ANOVA was used for statistical analysis. *P < 0.05, **P < 0.01; vs. 50% EtOH alone.

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**Immunohistochemical localization of 5-lipoxygenase in rat gastric mucosa.** Immunohistochemical studies reveal that immunoreactive 5-lipoxygenase positive cells were localized in the interstitial spaces of the gastric mucosa (Fig. 8, A and B). These positive cells were strongly stained with toluidine blue (data not shown), suggesting that these cells may be mast cells.

**Effects of LT antagonist and 5-lipoxygenase inhibitor on ethanol-induced gastric mucosal injury.** To observe the function of LTC₄ released by ethanol, we administered an LT antag-
shown that the expression of EP₂ mRNA could be detected in gastric mucosal cells; however, expression of EP₂ mRNA was not detected in any types of cells from the stomach by this method (27), although it was recently reported (21) that EP₂ is expressed in the stomach of the mouse, using RNase protection assay. Direct protective actions, such as inhibition of apoptosis of PGE₂ have been reported (20), but, as shown here, the indirect actions of PGE₂ were protective of nonvasculature elements.

It was previously reported that LTC₄ was a major mediator of ethanol-induced mucosal injury (6, 18, 23, 34, 35, 49). The activity of LTC₄ in inducing CV constriction was confirmed in the present experiment, suggesting that LTC₄ was a critical factor for ethanol-induced mucosal injury. In fact, an antagonist for LTC inhibited the injury elicited by ethanol in a

other processes that affect nonvascular tissues. It is also important for the protective action of EP₂ signaling to take place at concentrations that do not affect vascular components.

EP receptors involved in the indirect protection of the gastric mucosa by PGE₂ have been revealed to have the following actions: inhibition of gastric motility and stimulation of bicarbonate secretion by PGE₂ are mediated by the EP₁/EP₃ receptors (43, 46); the stimulation of mucin production by PGE₂ is mediated by EP₄ receptors (15); the increase in gastric cytoprotection by PGE₂ is mediated by EP₃ receptors (3); and inhibition of acid secretion by PGE₂ is mediated by EP₂/EP₃ receptors (29, 48). However, at the present we have had no concrete evidence as to the role of EP receptors in terms of the prevention of congestion of the gastric mucosa by PGE₂. The present results clarified the novel mechanisms of the preventive actions of PGE₂ on the microcircular dysfunction in ethanol-induced gastric mucosal injury.

There is not much evidence that an EP₂/EP₄ expressing cellular component was present in the gastric mucosa. The expression of EP receptors in the gastrointestinal tract of the mouse has been examined by in situ hybridization studies where it was

Fig. 7. Changes in intragastric LTC₄ levels during 50% ethanol perfusion (A) and effects of intragastric perfusion of PGE₂ and selective EP agonists on gastric LTC₄ levels before and after 50% ethanol perfusion (B). After perfusion of PGE₂ or selective EP agonists, leukotriene (LT)C released for 6 min after the exposure of 50% ethanol was determined with a specific ELISA. The values are expressed as means ± SE from 6 rats. ANOVA was used for statistical analysis. **P < 0.01; vs. after application of 50% EtOH after physiological saline perfusion.

Fig. 8. Effects of LTC₄ on the diameter of arterioles, collecting venules, and venules in the basal part of the gastric mucosal microcirculation (A) and effects of simultaneous administration of ONO-1078 (B). LTC₄ and ONO-1078 were applied to the exposed muscularis mucosae via the window. Data are expressed as means ± SE from 6 rats. ANOVA was used for the statistical analysis. ***P < 0.001. LTC₄, leukotriene C₄; ONO, ONO-1078.
Previously (31), EP2/4 signaling links to the adenylate cyclase EP4 agonist are promising agents for treating gastric mucosal injury. The gastric mucosal mast cells that express 5-lipoxygenase may be the source of LTC4 that constricts mucosal tissues do PGD2 (11). The gastric mucosal mast cells may be the source of LTC4 that constrains mucus collecting and other venules. The mucosal localization of the mast cells expressing 5-lipoxygenase corresponded well with the locations of microcirculation, which can be protected with an antagonist for LT, judging from the results of the present immunohistochemical study (Fig. 9). Further, we clarified here that the mast cells may be regulated by EP2/4 signaling (Fig. 7), suggesting that the site of action of EP2/4 signaling is the mucosal mast cells that express 5-lipoxygenase. The presence of EP2/4 receptors on the mucosal mast cells was reported previously (31). EP2/4 signaling links to the adenylate cyclase activation (9, 30), and it was reported that the elevation in cAMP levels stabilizes the mast cell activation (16). The released LTC4 was active in induction of gastric mucosal injury, since ONO-1078, which antagonized LTC4, inhibited ethanol-induced gastric mucosal injury. The same was true of the 5-lipoxygenase inhibitor AA861.

In summary, the administration of an EP2 agonist and an EP4 agonist completely inhibited ethanol-induced constriction of collecting venules and other venules in our microcirculation experiments. Arterioles were not markedly dilated by these agonists at 100 nM, a concentration at which the agonists can protect the gastric mucosa from 50% ethanol. The elevated LTC4 levels elicited by ethanol were significantly reduced with an EP2 agonist or an EP4 agonist, whereas neither an EP1 agonist nor an EP3 agonist reduced the LTC4 levels. Also, LTC4 released by ethanol has a significant role in mucosal injury. These results suggest that EP2 and EP4 agonists inhibit ethanol-induced gastric mucosal injury through the inhibition of gastric generation of LTC4, and that an EP2 agonist and an EP4 agonist are promising agents for treating gastric mucosal injury.

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GASTRIC MUCOSAL PROTECTION BY EP2 AND EP4 SIGNALING


