Mild irritant prevents ethanol-induced gastric mucosal microcirculatory disturbances through actions of calcitonin gene-related peptide and PGI$_2$ in rats

Takeo Saeki, Takashi Ohno, Kazuhsira Kamata, Katsuharu Arai, Sumito Mizuguchi, Makoto Katori, Katsunori Saigenji, and Masataka Majima. Mild irritant prevents ethanol-induced gastric mucosal microcirculatory disturbances through actions of calcitonin gene-related peptide and PGI$_2$ in rats. Am J Physiol Gastrointest Liver Physiol 286: G68–G75, 2004; 10.1152/ajpgi.00538.2002.—Pretreatment with a mild irritant such as 1 M NaCl prevented ethanol-induced mucosal injury, which was abolished by indomethacin, suggesting involvement of endogenous PGs. With the use of intravital microscopy, we investigated the mechanism in microcirculation whereby a mild irritant prevents ethanol-induced mucosal injury. Microcirculation of the basal part of gastric mucosa in anesthetized rats was observed through a window with transillumination. Diameters of arterioles, collecting venules, and venules were measured with an electric microscler. One molar NaCl alone caused dilation of arterioles and constrictions of collecting venules and venules, which were inhibited by indomethacin. Ethanol (50%) applied to mucosa constricted collecting venules and venules but dilated arterioles. Constriction of collecting venules resulted in mucosal congestion. Pretreatment with 1 M NaCl inhibited ethanol-induced constrictions of collecting venules and venules, and administration of indomethacin or a calcitonin gene-related peptide (CGRP) antagonist, CGRP-(8–37), abolished elimination of constrictions. Topical application (1 nM–10 $\mu$M) of PGE$_2$ or beraprost sodium (a PGI$_2$ analog) to microvasculature markedly and dose-dependently dilated arterioles, whereas that of PGE$_3$, but not beraprost, slightly constricted collecting venules. Pretreatment of microvasculature with a nonvasoactive concentration of PGE$_2$ (100 nM) or beraprost (1 nM) completely inhibited ethanol-induced constrictions of collecting venules. The inhibitory effect of beraprost but not of PGE$_2$ was abolished by CGRP-(8–37) (37). Present results suggest that the mechanism whereby 1 M NaCl prevents ethanol-induced injury is elimination of constrictions of collecting venules and venules by CGRP whose release may be enhanced by PGI$_2$ but not by PGE$_2$.

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It is generally accepted that mechanisms of gastric mucosal protection and repair depend on an appropriate microcirculatory blood supply, which helps to orchestrate the defense mechanism at various levels of gastroduodenal mucosa (8). Disturbances in gastric mucosal microcirculation are thought to be an important cause of injury, and observation of microcirculation is therefore important for revealing the pathophysiology of that injury. We modified the intravital microscopy technique originally developed by Guth and Rosenberg (7a) to make possible direct observation from the serosal side of microcirculation located in the basal part of gastric mucosa (11, 12, 21, 25). We were able to observe not only branching of basal mucosal arterioles to form mucosal capillaries but also venules collecting from mucosal surface and the following set of venules located in basal mucosa (11, 12, 21, 25). With the use of this technique, we revealed that a necrotizing agent, ethanol, induced marked dilatation of arterioles together with severe constriction of collecting venules and venules, and that constriction of these latter vessels played a part in mucosal damage as a result of congestion (21, 25).

It is well known that prior treatment with mild irritants, such as low concentrations of ethanol, taurocholate solution, and hypertonic solutions of NaCl prevented gastric mucosal injury produced by necrotizing agents, such as absolute ethanol. This phenomenon has been termed “adaptive cytoprotection” (5, 23, 24). Adaptive cytoprotection is thought to be mediated by increased production of gastric endogenous PGs as a result of induction by a mild irritant (24). We (19) have previously reported that 1 M NaCl administered into the stomach as a mild irritant increased production of PGE$_2$ and PGI$_2$ in the gastric wall and inhibited motility of gastric smooth muscle and development of ethanol-induced gastric mucosal lesions. Other reports emphasized that such adaptive cytoprotection by a mild irritant is largely attributable to increased mucosal blood flow, which dilutes necrotizing agents to which the stomach is exposed and proton ions diffused from the lumen of the stomach (13). Thus if to keep the integrity of mucosal microcirculation in response to mild irritant application is important in mucosal protection, observation of mucosal microcirculation with intravital microscopy is surely a useful maneuver for investigating how this protective action takes place during exposure to a mild irritant.

It has been indicated that capsaicin inhibits ethanol-induced gastric mucosal injury (9), a preventive effect that is termed a neural emergency system, and was attributable mainly to calcitonin gene-related peptide (CGRP) released from primary afferent neurons (7, 16). Previous reports have shown that endogenous PGs have also been implicated in capsaicin-sen-
sitive neuron-mediated cytoprotection (17, 27). We (3) previously reported that preperfusion with 1 M NaCl increased generation of gastric prostaglandins and reduced ethanol-induced mucosal damage. Following this treatment, intragastric CGRP levels increased, and protective action of 1 M NaCl was inhibited by a specific CGRP antagonist (3). These findings strongly suggested that the mechanism of adaptive cytoprotection exhibits similarities to that of protection by capsaicin.

In the present study, we investigated the mechanism of adaptive cytoprotection of 1 M NaCl against ethanol by observing microcirculation in the basal part of gastric mucosa of rats using intravital microscopy (25) to clarify mediators involved in maintenance of integrity of mucosal blood flow.

MATERIALS AND METHODS

Observation of rat gastric microcirculation. Male Sprague Dawley rats (specific pathogen free; 300–450 g) obtained from 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 experiments but had free access to water. Mucosal microcirculation of rats anesthetized with urethane (Alidrich, Milwaukee, WI) (0.875 g/kg ip) was observed through a modification improving on the method originally reported by Rosenberg and Guth (7a). After laparotomy with an electric cautery scalpel (model B-365; Takahashi Shoten, Tokyo, Japan), the greater curvature of the stomach was incised longitudinally exposing 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. A small part of the serosa, together with smooth muscle layers and submucosa was carefully dissected away using microsurgical scissors under a stereomicroscope. This method made possible direct observation of microcirculation of the basal part of gastric mucosa. Rectal temperature was continuously monitored by an electrical thermometer (Thermistor model MGA-3 type 219; Nihon Kohden, Tokyo, Japan) and was maintained at 37–38°C with a heating lamp. Systolic blood pressure was monitored by a pressure transducer (model TP200-T; Nihon Kohden) through a PE-50 tube inserted into the left femoral artery. Rats with a systolic blood pressure <90 mmHg or with a body temperature <37°C were discarded. At the end of the study, animals were killed by intravenous (iv) injection of an overdose of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL) in accordance with stipulations of the Experimental Animal Manual of the Kitasato University School of Medicine, Kanagawa, Japan.

Microcirculation of the basal part of mucosa was examined with a light microscope (Optiphot-2; Nikon, Tokyo, Japan) with a long working distance objective lens (M plan 10/0.21 SLWD; Nikon). Images of microcirculation were transmitted through a monochrome TV camera (C-2400 and C2400-7; Hamamatsu Photonics, Hamamatsu, Japan) to a TV monitor screen (model TM 1550; Ikegami Tsushinki, Tokyo Japan) and recorded with a videotape recorder (model BR-S800; Victor, Tokyo, Japan). One arteriole, one venule, and two collecting venules were selected in one observation window, and their internal diameters were measured using an adjustable electronic microscaler (model Argus-10; Hamamatsu Photonics). Diameter measurements were expressed as percentages of the original diameter.

Drug application. Ethanol (Wako Pure Chemical, Osaka, Japan); NaCl (Wako Pure Chemical); CGRP-(8–37) (10 μM; 20 μl), PGE2 (1 nM–1 μM; 20 μl), and beraprost sodium (1 nM–10 μM; 20 μl) were applied in the window. Indomethacin (1 mg/kg; Banyu Pharmaceutical, Tokyo, Japan) was administered intravenously.

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

RESULTS

Microcirculatory changes in the basal part of gastric mucosa after exposure of mucosa to 50% ethanol. Figure 1A shows microvasculature of the basal part of gastric mucosa before exposure to ethanol when observed through the window from the serosal side. Arterioles (10–45 μm in diameter) gave rise to capillaries that carried blood toward mucosa. Collecting venules with a diameter of 35–78 μm, which descended from the mucosal surface and were seen in cross section, joined venules (38–75 μm in diameter), which were the same size as collecting venules to which they were connected. Venules ran through the basal part of mucosa parallel to muscularis mucosa.

Gastric mucosa was exposed for 3 min to 50% ethanol, which was then washed out with Tyrode’s solution. Application of 50% ethanol on the mucosal side caused marked dilatation of arterioles, but collecting venules and venules were strongly constricted with the internal cross section of the collecting venules, in particular becoming very small as shown in Fig. 1B. Maximum dilatation of arterioles (247.0 ± 7.6%) occurred 7 min after application of ethanol (Fig. 2), and maximum constriction of collecting venules (56.7 ± 6.5%) and venules (60.0 ± 9.6%) occurred 3 min after this exposure (Fig. 2). Both of these rapid venular constrictions were followed by some dilatation when ethanol was washed off, but the vessels did not return to their basal resting size (Fig. 2). Constriction of venules persisted longer than 1 h.

Above changes in the microcirculation may explain our previous results (3), which indicated that 50% ethanol caused reddening and mucosal damage that resulted in congestion of mucosa.

Microcirculatory changes induced by 1 M NaCl in the basal part of gastric mucosa and effects of indomethacin pretreatment. A mild irritant (1 M NaCl) was applied to the mucosal side of the stomach wall for 10 min and was then washed out. Exposure of mucosa with 1 M NaCl dilated arterioles markedly. The time courses of changes are presented in Fig. 3 (solid lines). Maximum dilatation (274.1 ± 30.7%) occurred within 6 min after application. Application of 1 M NaCl caused slight constriction of collecting venules and venules 8 and 6 min after exposure, respectively (Fig. 3, solid line).

To investigate whether or not 1 M NaCl-induced microvascular changes were due to generation of endogenous PGs, we infused indomethacin (10 mg/kg iv) 10 min before application of 1 M NaCl. As shown in Fig. 3 (dotted line), 1 M NaCl-induced arteriolar dilatation was strongly inhibited by preapplication of indomethacin. Constriction of collecting venules and venules was also blocked by indomethacin (Fig. 3, dotted line).

These findings suggest that microvascular changes induced by 1 M NaCl were largely dependent on endogenous prostaglandins generated.
Effect of prior administration of 1 M NaCl on 50% ethanol-induced microcirculatory changes in the basal part of gastric mucosa. Figure 1C shows microvasculature of the basal part of gastric mucosa before exposure to 1 M NaCl and 50% ethanol when observed through the window from the serosal side. When 1 M NaCl was applied to the window 5 min before mucosal treatment with 50% ethanol, ethanol-induced rapid constrictions of the collecting venules and venules were completely inhibited by this preapplication (Figs. 1D and 2, solid line), although 1 M NaCl alone caused some constriction of both collecting venules and venules as mentioned above (Fig. 3). The diameter of arterioles during ethanol exposure was not affected by prior administration of 1 M NaCl, although they were markedly dilated during 1 M NaCl treatment (Fig. 2).

Furthermore, to investigate whether or not this preventive effect exerted by 1 M NaCl was attributable to release of endogenous PGs, indomethacin (10 mg/kg ic) was infused before the application of 1 M NaCl (Fig. 4, closed circles). Ethanol-induced constriction of collecting venules, which had been blocked by 1 M NaCl pretreatment, appeared again in rats treated with indomethacin (Fig. 4, closed circles). Dilatation of arterioles observed during ethanol exposure was not affected even by indomethacin preapplication (data not shown; 4 observations).

We then applied CGRP-(8–37) (10 μM), a CGRP receptor antagonist to microvasculature 3 min before treatment of 1 M NaCl to investigate involvement of endogenous CGRP (Fig. 4, open triangles). Treatment with CGRP-(8–37) did not affect size of microvessels under 1 M NaCl application, but ethanol-induced constriction of collecting venules blocked by 1 M NaCl pretreatment appeared again on treatment with a CGRP antagonist (Fig. 4, open triangles). Dilatation of arterioles observed during ethanol exposure was not affected by applications of CGRP-(8–37) (data not shown; 4 observations).

These results were similar to those obtained from indomethacin-pretreated rats.

Thus it is suggested that the protective effect of 1 M NaCl against ethanol-induced mucosal injury was mediated by both endogenous PGs and CGRP.

Effects of administration of PGs (PGE2 or beraprost) on ethanol-induced changes in microcirculation of basal part of gastric mucosa. Administration of PGE2 (1 nM–10 μM) or beraprost (1 nM–10 μM) to the observation window caused a rapid and dose-dependent dilatation of arterioles. Time courses of changes are presented in Fig. 5. Maximum dilatation of arterioles was observed within 1 min after PGE2 application. There was a prolonged dilatation of arterioles after beraprost application (Fig. 5). Dose-response curves at the time of maximum dilation are summarized in Fig. 5.

Since collecting venules are located upstream of venules, it is important to see changes in diameter of collecting venules. Figure 6 shows changes in diameter of collecting venules after administration of PGE2 (at final concentration; 1 nM–10 μM) or beraprost (at final concentration; 1 nM–10 μM) to the window. PGE2 caused a dose-dependent constriction of collecting venules, but beraprost induced no changes in diameter of collecting venules. The dose-response curve is shown in Fig. 6C.

Effect of CGRP-(8–37) on PG (PGE2 or beraprost)-induced inhibition of constriction of collecting venules by ethanol. To identify PGs contributing to 1 M NaCl-induced protective action, PGE2 or beraprost was applied to the window 3 min before the mucosal application of 50% ethanol. Low doses of PGE2 or beraprost which did not influence the original size of gastric mucosal vessels including arterioles are given. Both PGE2 (100 nM) and beraprost (1 nM) inhibited ethanol-induced rapid constrictions of collecting venules (Figs. 7C and 8B). Dilatation of arterioles observed during ethanol exposure...
was not affected by applications of these doses of PGE₂ and beraprost (Figs. 7A and 8A).

To test whether or not preventive effects of PGE₂ and beraprost were attributable to increased release of endogenous CGRP, a sufficient dose of CGRP-(8–37) (10 μM) was applied to the window 3 min before application of PGs (PGE₂: 100 nM or beraprost: 1 nM). Blockade of ethanol-induced constriction of collecting venules by beraprost appeared again with use of CGRP-(8–37) (Fig. 9B). By contrast, that by PGE₂ did not appear even with CGRP-(8–37) (Fig. 9B). These suggested that beraprost protected gastric mucosa from ethanol through cancellation of constriction of collecting venules utilizing CGRP, but PGE₂ did offer protection independently without CGRP.

DISCUSSION

In the present study, we found first that the mechanism of prevention by 1 M NaCl of ethanol-induced gastric mucosal injury is inhibition of constriction of collecting venules and venules via endogenous PGs and CGRP. Second, it was seen that PGI₂ analog, which did not dilate arterioles, collecting venules, or venules themselves, inhibited ethanol-induced constriction of collecting venules and venules suggesting that endogenous PGI₂ was responsible for the preventive effect seen when 1 M NaCl was applied before ethanol-induced injury through increased release of CGRP.
It was known that ingestion of ardent spirits caused erythema and morbid changes in human gastric mucosa (2). A stomach with no blood flow was reported to develop extensive mucosal damage within a very short period of time after contact with absolute ethanol. By contrast, an intact blood supply would afford a significant degree of protection from ethanol (1, 10, 14), indicating importance of blood flow in mucosal protection. Vascular injury with increased vascular permeability and endothelial cell damage is reported to be an early pathogenic change after ethanol administration (26).

Thus maintenance of blood flow may be a major protective factor. Erythema may be caused by increased blood flow due to arteriolar dilatation; however, ethanol-induced submucosal venular constriction associated with submucosal arteriolar dilatation was shown to be responsible for congestion and stasis (20). An early intravital microscopic study (4) reported that in ethanol-induced gastric mucosal injury, submucosal venular constriction was observed first, followed by cessation of mucosal blood flow and later by mucosal necrosis. Our findings in the present experiment together with our previous histological examination (11) confirmed that ethanol-induced mucosal injury is developed by congestion as a result of a rapid constriction of collecting venules. Outflow from the mucosa was firmly blocked at constricted collecting venules. Constriction of the basal part of the collecting venules and arteriolar dilatation as observed from the mucosal side was quite consistent with conditions characterized as mucosal congestion. We (12) have previously reported that congestion of mucosal blood flow is attributable to generation of leukotriene C4 (LTC4), which constricts collecting venules either directly or as a result of constriction of lamina muscularis mucosa, because topical application of LTC4 to the basal part of mucosal microcirculation induces pronounced constriction in venules and collecting venules. Our previous report (12) also stated that after exposure of the stomach to 30% ethanol for 3 min, the immunoreactive LTC4 level increased to 5.5 ng/stomach (vehicle alone: 0.71–0.85 ng/stomach). We observed a marked arteriolar dilatation, which was reported to be mediated by adenosine (18).

We (3) previously reported that preapplication of 1 M NaCl inhibited ethanol-induced mucosal damage induced by ethanol.
Present results showed that collecting venule and venule constriction was effectively eliminated by $1 \text{ M NaCl}$ pretreatment, and this effect of NaCl was abolished by indomethacin pretreatment (Fig. 4), suggesting involvement of endogenous PGs in this preventive action. Many reports (13) suggested that PGs may be cytoprotective as a result of their enhancement of the gastric mucosal blood flow. But, judging from present effects on arteriolar dilation during $1 \text{ M NaCl}$ treatment and the ethanol exposure that followed, increased blood supply to mucosa due to arteriolar dilatation was not significant in this protective action of NaCl. The present study clearly showed that elimination of constriction of collecting venules and venules may be quite critical for this prevention.

When applied alone to mucosa, $1 \text{ M NaCl}$ caused slight constriction of collecting venules and venules, which was blocked by indomethacin (Fig. 3). Our previous report (22) revealed that constriction of collecting venules and venules may be explained by the action of endogenous PG, such as PGE$_2$ which may stimulate EP3 receptor signaling. By contrast, collecting venule constriction by ethanol was not enhanced by $1 \text{ M NaCl}$ pretreatment but was inhibited instead. Switching of the effect of $1 \text{ M NaCl}$ on collecting venules and venules can be seen during mucosal exposure to ethanol. Therefore, other factors, such as neural factors, may be involved during ethanol exposure after NaCl application.

It is well known that prostanoids enhance pain sensation produced by chemical or mechanical stimulation. Recent reports (17, 26) have shown that endogenous PGs have also been implicated in the capsaicin-sensitive neuron-mediated neural emergency system. We previously reported increased release of CGRP during exposure to ethanol when endogenous PG formation was facilitated (3). Then, to investigate whether or not a preventive effect of $1 \text{ M NaCl}$ was attributable to released PGE$_2$ and beraprost sodium on the diameters of collecting venules in the basal part of the mucosal microcirculation. Each value indicates mean ± SE.

![Fig. 6. Effects of PGE$_2$ and beraprost sodium on the diameters of collecting venules in the basal part of the mucosal microcirculation. Each value indicates mean ± SE.](image)

![Fig. 7. Effect of PGE$_2$ on ethanol-induced changes in diameters of arterioles (A) and collecting venules (B). PGE$_2$ was administered prior to the mucosal application of 50% ethanol. Constriction of the collecting venules was inhibited by the treatments. Each value indicates mean ± SE. *P < 0.05.](image)
CGRP in response to PG generation in microvasculature, we applied a CGRP antagonist, CGRP-(8–37). The inhibitory effect of 1 M NaCl on ethanol-induced venular constriction was completely abolished, and ethanol-induced venular constriction appeared again (Fig. 4). These results strongly suggested that PGs generated by 1 M NaCl enhanced release of CGRP from primary sensory neurons on exposure to ethanol.

Leung et al. (15) proposed that nonantisecretory cytoprotective doses of PGs reduced resting gastric mucosal blood flow. In this study, ethanol-induced collecting venular constriction was inhibited by topical PGE2 (100 nM) or PGI2 (1 nM) (Figs. 7 and 8). These doses themselves did not influence original size of gastric mucosal vessels, including arterioles (Fig. 6). Thus our above results also supported the premise that the cytoprotective effect of PGs against ethanol-induced venular constriction was attributable to other factors, not simply to an increase of mucosal blood flow due to arteriolar dilatation. To investigate whether or not this preventive effect of PGs (100 nM PGE2 or 1 nM PGI2) took place through increased release of CGRP, CGRP-(8–37) was applied to the window (Fig. 9). Ethanol-induced constriction of collecting venules, which had been blocked by PGI2 pretreatment, appeared again with CGRP-(8–37), but this did not show any effect on PGE2-induced prevention of collecting venule constriction. Our previous studies using perfusion experiments showed that endogenous PGI2 generation, but not PGE2 generation, may participate in sensitization of CGRP-containing nerves, judging from release of CGRP when these PGs were administered (3). Furthermore, since beraprost-induced prevention of collecting venule constriction was abolished with CGRP-(8–37), PGI2 may play an important role in adaptive cytoprotection of 1 M NaCl by releasing CGRP from capsaicin-sensitive neurons. On the other hand, PGE2-mediated protection may occur through the CGRP-independent pathway, which includes inhibition of acid secretion, motility, increase of mucin secretion, and suppression of various cellular immune functions (6). Alternatively, since the venular constriction by ethanol was initiated by leukotrienes generated from mucosal mast cells (11), PGE2 may inhibit release of leukotrienes from mast cells.

Fig. 8. Effect of beraprost sodium on ethanol-induced changes in diameters of arterioles and collecting venules. Beraprost sodium was administered prior to the mucosal application of 50% ethanol. Constrictions of collecting venules were inhibited with treatments. Each value indicates mean ± SE. *P < 0.05.

Fig. 9. Effect of CGRP-(8–37) on PGE2 (A)- or beraprost sodium (B)-induced cancellation of constrictions of collecting venules by ethanol. CGRP-(8–37) and PGE2/beraprost sodium were administered prior to the mucosal application of 50% ethanol. Constrictions of collecting venules reappeared with CGRP-(8–37) treatments in rats after treatment with beraprost sodium, but not with PGE2. Each value indicates mean ± SE. *P < 0.05.
In conclusion, present results suggest that inhibition of ethanol-induced gastric injury by 1 M NaCl is attributable to suppression of collecting venule constriction by release of CGRP, which may be enhanced by PGL. The dilator of collecting venules and venules or both may in future become a useful agent for preventing induction of gastric mucosal injury by various necrotizing agents such as ethanol.

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